CORRECTED
VERSION*

**PCT**

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|---|---|
| (51) International Patent Classification 6 :<br><br>A61K 49/00 | A1 | (11) International Publication Number: **WO 97/18841**<br><br>(43) International Publication Date: 29 May 1997 (29.05.97) |

(21) International Application Number: PCT/US95/15040

(22) International Filing Date: 17 November 1995 (17.11.95)

(71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; Suite 350, 900 Welch Road, Palo Alto, CA 94304 (US).

(72) Inventors: CONTAG, Christopher, H.; 6110 Bollinger Road, San Jose, CA 95129 (US). CONTAG, Pamela, R.; 6110 Bollinger Road, San Jose, CA 95129 (US). BENARON, David, A.; 454 Birch Street, Redwood City, CA 94062 (US).

(74) Agents: HALLUIN, Albert, H. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AL, AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: NON-INVASIVE LOCALIZATION OF A LIGHT-EMITTING CONJUGATE IN A MAMMAL

(57) Abstract

Methods and compositions for detecting and localizing light originating from a mammal are disclosed. Also disclosed are methods for targeting light emission to selected regions, as well as for tracking entities within the mammal. In addition, animal models for disease states are disclosed, as are methods for localizing and tracking the progression of disease or a pathogen within the animal, and for screening putative therapeutic compounds effective to inhibit the disease or pathogen.

AP

# Non-Invasive Localization of a Light-Emitting
## Conjugate in a Mammal

5 FIELD OF THE INVENTION

The present invention relates to noninvasive methods and compositions for detecting, localizing and tracking light-emitting entities and biological events in a mammalian subject.

10

BACKGROUND OF THE INVENTION

The ability to monitor the progression of infectious diseases is limited by the current *ex vivo* methods of detecting and quantifying infectious agents in tissues. The 15 replication of an infectious agent in a host often involves primary, secondary and tertiary sites of replication. The sites of replication and the course that an infectious agent follows through these sites is determined by the route of inoculation, factors encoded by the host as well as 20 determinants of the infecting agent.

Experience may offer, in some cases, an estimate of probable sites of replication and the progress of an infection. It is more often the case, however, that the sites of infection, and the pace of the disease are either 25 not known or can only roughly be estimated. Moreover, the progression of an infectious disease, even in inbred strains of mice, is often individualized, and serial, *ex vivo* analyses of many infected animals need to be conducted to determine, on the average, what course a disease will follow 30 in an experimentally infected host.

Accordingly, it would be desirable to have a means of tracking the progression of infection in an animal model. Ideally, the tracking could be done non-invasively, such that a single animal could be evaluated as often as necessary 35 without detrimental effects. Methods and compositions of the present invention provide a non-invasive approach to detect,

localize and track a pathogen, as well as other entities, in
a living host, such as a mammal.

SUMMARY OF THE  INVENTION

5       In one embodiment, the invention includes a noninvasive
method for detecting the localization of a biocompatible
entity in a mammalian subject.  The entity can be a molecule,
macromolecule, cell, microorganism (including a pathogen), a
particle, or the like.

10      The method includes administering to the subject a
conjugate of the entity and a light-generating moiety.
Light-generating moieties are typically molecules or
macromolecules that give off light.  They may generate light
as a result of radiation absorption (e.g. fluorescent or

15 phosphorescent molecules), or as a result of a chemical
reaction (e.g. bioluminescent proteins).  Exemplary light-
generating moieties are bioluminescent proteins, such as
luciferase and aequorin, and colored or fluorescent proteins,
such as yellow fluorescent protein and ferredoxin IV.

20      The moiety may be conjugated to the entity by a variety
of techniques, including incorporation during synthesis of
the entity (e.g. chemical or genetic, such a fusion protein
of an antibody fragment and a light-generating protein),
chemical coupling post-synthesis, non-covalent association

25 (e.g. encapsulation by liposomes), in-situ synthesis in the
entity (e.g. expression of a heterologous bioluminescent
protein in a transformed cell), or in situ activatable
promoter-controlled expression of a bioluminescent protein in
cells of a transgenic animal stimulated by a promoter inducer

30 (e.g. interferon-activated promoter stimulated by infection
with a virus).

        After a period of time in which the conjugate can
localize in the subject, the subject is immobilized within
the detection field of a photodetector device for a period of

35 time effective to measure a sufficient amount of photon
emission (with the photodetector device) to construct an
image.  An exemplary photodetector device is an intensified

charge-coupled device (ICCD) camera coupled to an image
processor. If the image can be constructed in a time short
relative to the time scale at which an "unimmobilized"
subject moves, the subject is inherently "immobilized" during
5   imaging and no special immobilization precautions are
required. An image from the photon emission data is then
constructed.

The method described above can be used to track the
localization of the entity in the subject over time, by
10  repeating the imaging steps at selected intervals and
constructing images corresponding to each of those intervals.

The method described above can be used in a number of
specific applications, by attaching, conjugating or
incorporating targeting moieties onto the entity. The
15  targeting moiety may be an inherent property of the entity
(e.g. antibody or antibody fragment), or it may be conjugated
to, attached to, or incorporated in the entity (e.g.
liposomes containing antibodies). Examples of targeting
moieties include antibodies, antibody fragments, enzyme
20  inhibitors, receptor-binding molecules, various toxins and
the like. Targets of the targeting moiety may include sites
of inflammation, infection, thrombotic plaques and tumor
cells. Markers distinguishing these targets, suitable for
recognition by targeting moieties, are well known.
25      Further, the method may be used to detect and localize
sites of infection by a pathogen in an animal model, using
the pathogen (e.g. Salmonella) conjugated to a light-
generating moiety as the entity.

In a related embodiment, the invention includes a
30  noninvasive method for detecting the level of a biocompatible
entity in a mammalian subject over time. The method is
similar to methods described above, but is designed to detect
changes in the level of the entity in the subject over time,
without necessarily localizing the entity in the form of an
35  image. This method is particularly useful for monitoring the
effects of a therapeutic substance, such an antibiotic, on

the levels of an entity, such as a light-emitting bacterium, over time.

In another embodiment, the invention includes a noninvasive method for detecting the integration of a
5 transgene in a mammalian subject. The method includes administering to the subject, a vector construct effective to integrate a transgene into mammalian cells. Such constructs are well known in the art. In addition to the elements necessary to integrate effectively, the construct contains a
10 transgene (e.g. a therapeutic gene), and a gene encoding a light-generating protein under the control of a selected activatable promoter. After a period of time in which the construct can achieve integration, the promoter is activated. For example, if an interferon promoter is used, a poly-
15 inosine and -cytosine duplex (poly-IC) can be locally administered (e.g. footpad injection) to stimulate interferon production. The subject is then placed within the detection field of a photodetector device, such as an individual wearing light-intensifying "night vision" goggles, and the
20 level of photon emission is measured, or evaluated. If the level is above background (i.e. if light can be preferentially detected in the "activated" region), the subject is scored as having integrated the transgene.

In a related embodiment, the invention includes a
25 noninvasive method for detecting the localization of a promoter-induction event in an animal made transgenic or chimeric for a construct including a gene encoding a light-generating protein under the control of an inducible promoter. Promoter induction events include the
30 administration of a substance which directly activates the promoter, the administration of a substance which stimulates production of an endogenous promoter activator (e.g. stimulation of interferon production by RNA virus infection), the imposition of conditions resulting in the production of
35 an endogenous promoter activator (e.g. heat shock or stress), and the like. The event is triggered, and the animal is imaged as described above.

- 4 -

In yet another embodiment, the invention includes
pathogens, such as *Salmonella*, transformed with a gene
expressing a light-generating protein, such as luciferase.

In another aspect, the invention includes a method of
5 identifying therapeutic compounds effective to inhibit spread
of infection by a pathogen. The method includes
administering a conjugate of the pathogen and a light-
generating moiety to control and experimental animals,
treating the experimental animals with a putative therapeutic
10 compound, localizing the light-emitting pathogen in both
control and experimental animals by the methods described
above, and identifying the compound as therapeutic if the
compound is effective to significantly inhibit the spread or
replication of the pathogen in the experimental animals
15 relative to control animals. The conjugates include a
fluorescently-labeled antibodies, fluorescently-labeled
particles, fluorescently-labeled small molecules, and the
like.

In still another aspect, the invention includes a method
20 of localizing entities conjugated to light-generating
moieties through media of varying opacity. The method
includes the use of photodetector device to detect photons
transmitted through the medium, integrate the photons over
time, and generate an image based on the integrated signal.
25      In yet another embodiment, the invention includes a
method of measuring the concentration of selected substances,
such as dissolved oxygen or calcium, at specific sites in an
organism. The method includes entities, such as cells,
containing a concentration sensor - a light-generating
30 molecule whose ability to generate light is dependent on the
concentration of the selected substance. The entity
containing the light-generating molecule is administered such
that it adopts a substantially uniform distribution in the
animal or in a specific tissue or organ system (*e.g.* spleen).
35 The organism is imaged, and the intensity and localization of
light emission is correlated to the concentration and
location of the selected substance. Alternatively, the

- 5 -

entity contains a second marker, such as a molecule capable
of generating light at a wavelength other than the
concentration sensor.  The second marker is used to normalize
for any non-uniformities in the distribution of the entity in
5 the host, and thus permit a more accurate determination of
the concentration of the selected substance.

In another aspect, the invention includes a method of
identifying therapeutic compounds effective to inhibit the
growth and/or the metastatic spread of a tumor.  The method
10 includes (i) administering tumor cells labeled with or
containing light-generating moieties to groups of
experimental and control animals, (ii) treating the
experimental group with a selected compound, (iii) localizing
the tumor cells in animals from both groups by imaging photon
15 emission from the light-generating molecules associated with
the tumor cells with a photodetector device, and (iv)
identifying a compound as therapeutic if the compound is able
to significantly inhibit the growth and/or metastatic spread
of the tumor in the experimental group relative to the
20 control group.

These and other objects and features of the invention
will be more fully appreciated when the following detailed
description of the invention is read in conjunction with the
25 accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES
     Figures 1A, 1B and 1C show a map of the lux pCGLS1
plasmid used to transform Salmonella strains SL1344, BJ66 and
30 LB5000 to generate strains SL1344lux, BJ66lux and LB5000lux.
     Figures 2A-E show the results of assays to measure
adherence and invasion, by Salmonella strains SL1344lux and
BJ66lux, of macrophages and HEp-2 cells.  Figure 2A shows
luminescent bacterial cells localized in wells of an assay
35 dish.  The pseudo-color image, obtained by integrating
photons over one minute, is superimposed over a gray scale
image of the assay dish, producing the "composite image"

shown. Figure 2B shows the relative light intensity of wells
that were not treated with gentamicin. Figure 2C shows the
number of colony forming units (CFU) per ml isolated from the
same wells as were imaged in Figure 2B. Figure 2D shows the
5 relative light intensity of wells that were treated with
gentamicin. Figure 2E shows the number of colony forming
units (CFU) per ml isolated from the same wells as were
imaged in Figure 2D.

Figure 3A is a composite image of four glass capillary
10 tubes containing dilutions of LB5000lux bacterial
suspensions. Luminescence was determined by integrating over
30 seconds. Air pockets are present in each tube on both
sides of the suspension.

Figure 3B depict the distribution of bioluminescence
15 following intraperitoneal inoculation of wild-type SL1344lux
into mice.

Figure 4 is a schematic diagram of a vial used to test
the transmission of light generated by LB5000lux through
animal tissue.

20      Figures 5A-F show composite images of Balb/c mice orally
inoculated with low virulence LB5000lux (Figs. 5A-B), non-
invasive BJ66lux (Figs. 5C-D) and virulent SL1344lux (Figs.
5E-F) Salmonella, and imaged at the times indicated in the
figure. The luminescence signal was integrated over 5
25 minutes.

Figure 6 is a composite image showing the distribution
of Salmonella in mice 32 hours following intraperitoneal
(i.p.) injections with either virulent SL1344lux (left two
animals) or low virulence LB5000lux (right two animals)
30 strains of the bacterium.

Figures 7A and 7B show the distribution of virulent
Salmonella in mice resistant to systemic Salmonella
infections (129 x Balb/c, $Ity^{r/s}$). Figure 7A - day 1, Figure
7B - day 8.

35      Figures 8A-C show the distribution of mutant Salmonella
with reduced virulence (BJ66lux) seven days following oral
inoculation. Figure 8A shows external, non-invasive imaging

- 7 -

of the luminescence.  Figure 8B shows the same animal imaged
following laparotomy.  Labeled organs are C - cecum, L -
liver, I - small intestine and Sp - spleen.  Figure 8C shows
a post-laparotomy image generated following injection of air
5 into the lumen of the intestine both anterior and posterior
to the cecum.

    Figures 9A, 9B and 9C show the distribution of
Salmonella SL1344lux in susceptible Balb/c mice following
intraperitoneal inoculation with SL1344lux.  Figure 9A was
10 imaged prior to the opening of the peritoneal cavity.  Figure
9B was imaged after the opening of the peritoneal cavity, and
Figure 9C was imaged after the cecum was pulled to the left
side.

    Figures 10A-E show the effects of ciprofloxacin
15 treatment on bioluminescence from SL1344lux Salmonella in
orally-inoculated mice.  Figure 10A shows a graph of the
relative bioluminescence intensity, measured from the
abdominal area, as a function of time after initiation of
treatment, for treated and untreated animals.  Figures 10B
20 and 10D show composite images of mice 8 days after oral
inoculation with SL1344lux Salmonella, before treatment with
ciprofloxacin.  Figures 10C and 10E show composite images of
the same mice 5.5 hours either following treatment (Fig. 10E)
or control (no treatment; Fig. 10C).
25

DETAILED DESCRIPTION OF THE INVENTION

I.    Definitions
    Unless otherwise indicated, all terms used herein have
the same meaning as they would to one skilled in the art of
30 the present invention.
    Opaque medium is used herein to refer to a medium that
is "traditionally" opaque, not necessarily absolutely opaque.
Accordingly, an opaque medium is defined as a medium that is
commonly considered to be neither transparent nor
35 translucent, and includes items such as a wood board, and
flesh and skin of a mammal.

Luciferase, unless stated otherwise, includes
prokaryotic and eukaryotic luciferases, as well as variants
possessing varied or altered optical properties, such as
luciferases that luminesce at wavelengths in the red range.

5       Biocompatible entity is an entity that can be
administered to a mammal. This includes pathogens which may
be deleterious to the mammal. In reference to an animal
whose cells contain a transgene expressing a light-generating
protein, biocompatible entity refers to the transgene-
10 containing cells comprising the mammal.

Light-generating is defined as capable of generating
light through a chemical reaction or through the absorption
of radiation.

Light is defined herein, unless stated otherwise, as
15 electromagnetic radiation having a wavelength of between
about 300 nm and about 1100 nm.

Spread of infection typically refers to the spreading
and colonization by a pathogen of host sites other than the
initial infection site. The term can also include, however,
20 growth in size and/or number of the pathogen at the initial
infection site.

lux - prokaryotic genes associated with luciferase and
photon emission.

luc - eukaryotic genes associated with luciferase and
25 photon emission.

Promoter induction event refers to an event that results
in the direct or indirect induction of a selected inducible
promoter.

Heterologous gene refers to a gene which has been trans-
30 fected into a host organism. Typically, a heterologous gene
refers to a gene that is not originally derived from the
transfected or transformed cells' genomic DNA.

II.  General Overview of the Invention
35      The present invention includes methods and compositions
relating to non-invasive imaging and/or detecting of light-
emitting conjugates in mammalian subjects. The conjugates

- 9 -

contain a biocompatible entity and a light-generating moiety.
Biocompatible entities include, but are not limited to, small
molecules such as cyclic organic molecules; macromolecules
such as proteins; microorganisms such as viruses, bacteria,
5  yeast and fungi; eukaryotic cells; all types of pathogens and
pathogenic substances; and particles such as beads and
liposomes.  In another aspect, biocompatible entities may be
all or some of the cells that constitute the mammalian
subject being imaged.

10        Light-emitting capability is conferred on the entities
by the conjugation of a light-generating moiety.  Such
moieties include fluorescent molecules, fluorescent proteins,
enzymatic reactions giving off photons and luminescent
substances, such as bioluminescent proteins.  The conjugation
15  may involve a chemical coupling step, genetic engineering of
a fusion protein, or the transformation of a cell,
microorganism or animal to express a bioluminescent protein.
For example, in the case where the entities are the cells
constituting the mammalian subject being imaged, the light-
20  generating moiety may be a bioluminescent or fluorescent
protein "conjugated" to the cells through localized,
promoter-controlled expression from a vector construct
introduced into the cells by having made a transgenic or
chimeric animal.

25        Light-emitting conjugates are typically administered to
a subject by any of a variety of methods, allowed to localize
within the subject, and imaged.  Since the imaging, or
measuring photon emission from the subject, may last up to
tens of minutes, the subject is usually, but not always,
30  immobilized during the imaging process.

        Imaging of the light-emitting entities involves the use
of a photodetector capable of detecting extremely low levels
of light - typically single photon events - and integrating
photon emission until an image can be constructed.  Examples
35  of such sensitive photodetectors include devices that
intensify the single photon events before the events are
detected by a camera, and cameras (cooled, for example, with

- 10 -

liquid nitrogen) that are capable of detecting single photons over the background noise inherent in a detection system.

Once a photon emission image is generated, it is typically superimposed on a "normal" reflected light image of
5 the subject to provide a frame of reference for the source of the emitted photons (i.e. localize the light-emitting conjugates with respect to the subject). Such a "composite" image is then analyzed to determine the location and/or amount of a target in the subject.
10 The steps and embodiments outlined above are presented in greater detail, below.


III. Light-Emitting Entities
    A.    Light-Generating Moieties
15 The light-generating moieties (LGMs), molecules or constructs useful in the practice of the present invention may take any of a variety of forms, depending on the application. They share the characteristic that they are luminescent, that is, that they emit electromagnetic
20 radiation in ultraviolet (UV), visible and/or infra-red (IR) from atoms or molecules as a result of the transition of an electronically excited state to a lower energy state, usually the ground state.

Examples of light-generating moieties include
25 photoluminescent molecules, such as fluorescent molecules, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.

Two characteristics of LGMs that bear considerable relevance to the present invention are their size and their
30 spectral properties. Both are discussed in the context of specific types of light-generating moieties described below, following a general discussion of spectral properties.


        1.    Spectral Properties. An important aspect of
35 the present invention is the selection of light-generating moieties that produce light capable of penetrating animal tissue such that it can be detected externally in a non-

invasive manner. The ability of light to pass through a
medium such as animal tissue (composed mostly of water) is
determined primarily by the light's intensity and wavelength.

    The more intense the light produced in a unit volume,
5  the easier the light will be to detect. The intensity of
light produced in a unit volume depends on the spectral
characteristics of individual LGMs, discussed below, and on
the concentration of those moieties in the unit volume.
Accordingly, conjugation schemes that place a high
10 concentration of LGMs in or on an entity (such as high-
efficiency loading of a liposome or high-level expression of
a bioluminescent protein in a cell) typically produce
brighter light-emitting conjugates (LECs), which are easier
to detect through deeper layers of tissue, than schemes which
15 conjugate, for example, only a single LGM onto each entity.

    A second factor governing the detectability of an LGM
through a layer of tissue is the wavelength of the emitted
light. Water may be used to approximate the absorption
characteristics of animal tissue, since most tissues are
20 composed primarily of water. It is well known that water
transmits longer-wavelength light (in the red range) more
readily than it does shorter wavelength light.

    Accordingly, LGMs which emit light in the range of
yellow to red (550 - 1100 nm) are typically preferable to
25 LGMs which emit at shorter wavelengths. Several of the LGMs
discussed below emit in this range. However, it will be
noted, based on experiments performed in support of the
present invention and presented below, that excellent results
can be achieved in practicing the present invention with LGMs
30 that emit in the range of 486 nm, despite the fact that this
is not an optimal emission wavelength. These results are
possible, in part, due to the relatively high concentration
of LGMs (luciferase molecules) present in the LECs
(transformed *Salmonella* cells) used in these experiments, and
35 to the use of a sensitive detector. It will be understood
that through the use of LGMs with a more optimal emission

wavelength, similar detection results can be obtained with
LGEs having lower concentrations of the LGMs.


        2.   Fluorescence-based Moieties.   Fluor-escence is
5  the luminescence of a substance from a single electronically
excited state, which is of very short duration after removal
of the source of radiation.   The wavelength of the emitted
fluorescence light is longer than that of the exciting
illumination (Stokes' Law), because part of the exciting
10  light is converted into heat by the fluorescent molecule.
        Because fluorescent molecules require input of light in
order to luminesce, their use in the present invention may be
more complicated than the use of bioluminescent molecules.
Precautions are typically taken to shield the excitatory
15  light so as not to contaminate the fluorescence photon signal
being detected from the subject.   Obvious precautions include
the placement of. an excitation filter, such that employed in
fluorescence microscope, at the radiation source.   An
appropriately-selected excitation filter blocks the majority
20  of photons having a wavelength similar to that of the photons
emitted by the fluorescent moiety.   Similarly a barrier
filter is employed at the detector to screen out most of the
photons having wavelengths other than that of the
fluorescence photons.   Filters such as those described above
25  can be obtained from a variety of commercial sources,
including Omega Optical, Inc. (Brattleboro, VT).
        Alternatively, a laser producing high intensity light
near the appropriate excitation wavelength, but not near the
fluorescence emission wavelength, can be used to excite the
30  fluorescent moieties.   An x-y translation mechanism may be
employed so that the laser can scan the subject, for example,
as in a confocal microscope.
        As an additional precaution, the radiation source can be
placed behind the subject and shielded, such that the only
35  radiation photons reaching the site of the detector are those
that pass all the way through the subject.   Furthermore,

detectors may be selected that have a reduced sensitivity to wavelengths of light used to excite the fluorescent moiety.

Through judicious application of the precautions above, the detection of fluorescent LGMs according to methods of the
5 present invention is possible.

Fluorescent moieties include small fluorescent molecules, such as fluorescein, as well as fluorescent proteins, such as green fluorescent protein (Chalfie, et al., 1994, Science 263:802-805., Morin and Hastings, 1971, J.
10 Cell. Physiol. 77:313) and lumazine and yellow fluorescent proteins (O'Kane, et al., 1991, PNAS 88:1100-1104, Daubner, et al., 1987, PNAS 84:8912-8916). In addition, certain colored proteins such as ferredoxin IV (Grabau, et al., 1991, J Biol Chem. 266:3294-3299), whose fluorescence
15 characteristics have not been evaluated, may be fluorescent and thus applicable for use with the present invention. Ferredoxin IV is a particularly promising candidate, as it has a reddish color, indicating that it may fluoresce or reflect at a relatively long wavelength and produce light
20 that is effective at penetrating tissue. Furthermore, the molecule is small for a protein (95 amino acids), and can thus be conjugated to entities with a minimal impact on their function.

An advantage of small fluorescent molecules is that they
25 are less likely to interfere with the bioactivity of the entity to which they are attached than a would a larger light-generating moiety. In addition, commercially-available fluorescent molecules can be obtained with a variety of excitation and emission spectra that are suitable for use
30 with the present invention. For example, Molecular Probes (Eugene, OR) sells a number of fluorophores, including Lucifer Yellow (abs. at 428 nm, and emits at 535 nm) and Nile Red (abs. at 551 nm and emits at 636 nm). Further, the molecules can be obtained derivatized with a variety of
35 groups for use with various conjugation schemes (e.g. from Molecular Probes).

3.    Bioluminescence-based Moieties.  The subjects
of chemiluminescence (luminescence as a result of a chemical
reaction) and bioluminescence (visible luminescence from
living organisms) have, in many aspects, been thoroughly
5 studied (e.g., Campbell, 1988, Chemiluminescence.  Principles
and Applications in Biology and Medicine (Chichester,
England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft
mbH)).  A brief summary of salient features follows.
        Bioluminescent molecules are distinguished from
10 fluorescent molecules in that they do not require the input
of radiative energy to emit light.  Rather, bioluminescent
molecules utilize chemical energy, such as ATP, to produce
light.  An advantage of bioluminescent moieties, as opposed
to fluorescent moieties, is that there is virtually no
15 background in the signal.  The only light detected is light
that is produced by the exogenous bioluminescent moiety.  In
contrast, the light used to excite a fluorescent molecule
often results in the fluorescence of substances other than
the intended target.  This is particularly true when the
20 "background" is as complex as the internal environment of a
living animal.
        Several types of bioluminescent molecules are known.
They include the luciferase family (e.g. Wood, et al., 1989,
Science 244:700-702) and the aequorin family (e.g. Prasher,
25 et al., Biochem. 26:1326-1332).  Members of the luciferase
family have been identified in a variety of prokaryotic and
eukaryotic organisms.  Luciferase and other enzymes involved
in the prokaryotic luminescent (lux) systems, as well as the
corresponding lux genes, have been isolated from marine
30 bacteria in the Vibrio and Photobacterium genera and from
terrestrial bacteria in the Xenorhabdus genus.
        An exemplary eukaryotic organism containing a luciferase
system (luc) is the North American firefly Photinus pyralis.
Firefly luciferase has been extensively studied, and is
35 widely used in ATP assays.  cDNAs encoding luciferases from
Pyrophorus plagiophthalamus, another species of click beetle,
have been cloned and expressed (Wood, et al., 1989, Science

244:700-702). This beetle is unusual in that different
members of the species emit bioluminescence of different
colors. Four classes of clones, having 95-99% homology with
each other, were isolated. They emit light at 546 nm
5 (green), 560 nm (yellow-green), 578 nm (yellow) and 593 nm
(orange). The last class (593 nm) may be particularly
advantageous for use as a light-generating moiety with the
present invention, because the emitted light has a wavelength
that penetrates tissues more easily than shorter wavelength
10 light.

Luciferases, as well as aequorin-like molecules, require
a source of energy, such as ATP, NAD(P)H, and the like, and a
substrate, such as luciferin or coelentrizine and oxygen.
The substrate luciferin must be supplied to the
15 luciferase enzyme in order for it to luminesce. In those
cases where a luciferase enzyme is introduced as an
expression product of a vector containing cDNA encoding a *lux*
luciferase, a convenient method for providing luciferin is to
express not only the luciferase but also the biosynthetic
20 enzymes for the synthesis of luciferin. In cells transformed
with such a construct, oxygen is the only extrinsic
requirement for bioluminescence. Such an approach, detailed
in Example 1, is employed to generate *lux*-transformed
*Salmonella*, which are used in experiments performed in
25 support of the present invention and detailed herein.

The plasmid construct, encoding the *lux* operon obtained
from the soil bacterium *Xenorhabdus luminescens* (Frackman, *et
al.*, 1990, *J. Bact.* 172:5767-5773), confers on transformed *E
coli* the ability to emit photons through the expression of
30 the two subunits of the heterodimeric luciferase and three
accessory proteins (Frackman, *et al.*, 1990). Optimal
bioluminescence for *E. Coli* expressing the lux genes of *X.
luminescens* is observed at 37°C (Szittner and Meighen, 1990,
*J. Biol. Chem.* 265:16581-16587, Xi, *et al.*, 1991, *J. Bact.*
35 173:1399-1405) in contrast to the low temperature optima of
luciferases from eukaryotic and other prokaryotic luminescent
organisms (Campbell, 1988, Chemiluminescence. Principles and

- 16 -

Applications in Biology and Medicine (Chichester, England:
Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). The
luciferase from *X. luminescens*, therefore, is well-suited for
use as a marker for studies in animals.

5       Luciferase vector constructs such as the one described
above and in Example 1, can be adapted for use in
transforming a variety of host cells, including most
bacteria, and many eukaryotic cells (*luc* constructs). In
addition, certain viruses, such as herpes virus and vaccinia
10 virus, can be genetically-engineered to express luciferase.
For example, Kovacs Sz. and Mettenlieter, 1991, *J. Gen.
Virol.* 72:2999-3008, teach the stable expression of the gene
encoding firefly luciferase in a herpes virus. Brasier and
Ron, 1992, *Meth. in Enzymol.* 216:386-396, teach the use of
15 luciferase gene constructs in mammalian cells. Luciferase
expression from mammalian cells in culture has been studied
using CCD imaging both macroscopically (Israel and Honigman,
1991, *Gene* 104:139-145) and microscopically (Hooper, *et al.*,
1990, *J. Biolum. and Chemilum.* 5:123-130).
20

        B.    Entities
        The invention includes entities which have been modified
or conjugated to include a light-generating moiety, construct
or molecule, such as described above. Such conjugated or
25 modified entities are referred to as light-emitting entities,
light-emitting conjugates (LECs) or simply conjugates. The
entities themselves may take the form of, for example,
molecules, macromolecules, particles, microorganisms, or
cells. The methods used to conjugate a light-generating
30 moiety to an entity depend on the nature of the moiety and
the entity. Exemplary conjugation methods are discussed in
the context of the entities described below.


        1.    Small molecules. Small molecule entities
35 which may be useful in the practice of the present invention
include compounds which specifically interact with a pathogen
or an endogenous ligand or receptor. Examples of such

- 17 -

molecules include, but are not limited to, drugs or
therapeutic compounds; toxins, such as those present in the
venoms of poisonous organisms, including certain species of
spiders, snakes, scorpions, dinoflagellates, marine snails
5 and bacteria; growth factors, such as NGF, PDGF, TGF and TNF;
cytokines; and bioactive peptides.

The small molecules are preferably conjugated to light-
generating moieties that interfere only minimally, if at all,
with the bioactivity of the small molecule, such as small
10 fluorescent molecules (described above). Conjugations are
typically chemical in nature, and can be performed by any of
a variety of methods known to those skilled in the art.

The small molecule entity may be synthesized to contain
a light-generating moiety, so that no formal conjugation
15 procedure is necessary. Alternatively, the small molecule
entity may be synthesized with a reactive group that can
react with the light generating moiety, or *vice versa*.     -

Small molecules conjugated to light-generating moieties
of the present invention may be used either in animal models
20 of human conditions or diseases, or directly in human
subjects to be treated. For example, a small molecule which
binds with high affinity to receptor expressed on tumor cells
may be used in an animal model to localize and obtain size
estimates of tumors, and to monitor changes in tumor growth
25 or metastasis following treatment with a putative therapeutic
agent. Such molecules may also be used to monitor tumor
characteristics, as described above, in cancer patients.


2.    Macromolecules. Macromolecules, such as
30 polymers and biopolymers, constitute another example of
entities useful in practicing the present invention.
Exemplary macromolecules include antibodies, antibody
fragments, fusion proteins and certain vector constructs.

Antibodies or antibody fragments, purchased from
35 commercial sources or made by methods known in the art
(Harlow, *et al.*, 1988, Antibodies: A Laboratory Manual,
Chapter 10, pg. 402, Cold Spring Harbor Press), can be used

to localize their antigen in a mammalian subject by
conjugating the antibodies to a light-generating moiety,
administering the conjugate to a subject by, for example,
injection, allowing the conjugate to localize to the site of
5 the antigen, and imaging the conjugate.

   Antibodies and antibody fragments have several
advantages for use as entities in the present invention.  By
their nature, they constitute their own targeting moieties.
Further, their size makes them amenable to conjugation with
10 several types of light-generating moieties, including small
fluorescent molecules and fluorescent and bioluminescent
proteins, yet allows them to diffuse rapidly relative to, for
example, cells or liposomes.

   The light-generating moieties can be conjugated directly
15 to the antibodies or fragments, or indirectly by using, for
example, a fluorescent secondary antibody.  Direct
conjugation can be accomplished by standard chemical coupling
of, for example, a fluorophore to the antibody or antibody
fragment, or through genetic engineering.  Chimeras, or
20 fusion proteins can be constructed which contain an antibody
or antibody fragment coupled to a fluorescent or
bioluminescent protein.  For example, Casadei, et al., 1990,
PNAS 87:2047-2051, describe a method of making a vector
construct capable of expressing a fusion protein of aequorin
25 and an antibody gene in mammalian cells.

   Conjugates containing antibodies can be used in a number
of applications of the present invention.  For example, a
labeled antibody directed against E-selection, which is
expressed at sites of inflammation, can be used to localize
30 the inflammation and to monitor the effects of putative anti-
inflammatory agents.

   Vector constructs by themselves can also constitute
macromolecular entities applicable to the present invention.
For example, a eukaryotic expression vector can be
35 constructed which contains a therapeutic gene and a gene
encoding a light-generating molecule under the control of a
selected promoter (i.e. a promoter which is expressed in the

cells targeted by the therapeutic gene). Expression of the
light-generating molecule, assayed using methods of the
present invention, can be used to determine the location and
level of expression of the therapeutic gene. This approach
5 may be particularly useful in cases where the expression of
the therapeutic gene has no immediate phenotype in the
treated individual or animal model.


3.   Viruses. Another entity useful for certain
10 aspects of the invention are viruses. As many viruses are
pathogens which infect mammalian hosts, the viruses may be
conjugated to a light-generating moiety and used to study the
initial site and spread of infection. In addition, viruses
labeled with a light-generating moiety may be used to screen
15 for drugs which inhibit the infection or the spread of
infection.
     A virus may be labeled indirectly, either with an
antibody conjugated to a light-generating moiety, or by, for
example, biotinylating virions (e.g. by the method of Dhawan,
20 et al., 1991, J. Immunol. 147(1):102) and then exposing them
to streptavidin linked to a detectable moiety, such as a
fluorescent molecule.
     Alternatively, virions may be labeled directly with a
fluorophore like rhodamine, using, for example, the methods
25 of Fan, et al., 1992, J. Clin. Micro. 30(4):905. The virus
can also be genetically engineered to express a light-
generating protein. The genomes of certain viruses, such as
herpes and vaccinia, are large enough to accommodate genes as
large as the lux or luc genes used in experiments performed
30 in support of the present invention.
     Labeled virus can be used in animal models to localize
and monitor the progression of infection, as well as to
screen for drugs effective to inhibit the spread of
infection. For example, while herpes virus infections are
35 manifested as skin lesions, this virus can also cause herpes
encephalitis. Such an infection can be localized and
monitored using a virus labeled by any of the methods

described above, and various antiviral agents can be tested for efficacy in central nervous system (CNS) infections.

　　　　　4.　Particles.  Particles, including beads,
5 liposomes and the like, constitute another entity useful in the practice of the present invention.  Due to their larger size, particles may be conjugated with a larger number of light-generating molecules than, for example, can small molecules.  This results in a higher concentration of light
10 emission, which can be detected using shorter exposures or through thicker layers of tissue.  In addition, liposomes can be constructed to contain an essentially pure targeting moiety, or ligand, such as an antigen or an antibody, on their surface.  Further, the liposomes may be loaded with,
15 for example, bioluminescent protein molecules, to relatively high concentrations (Campbell, 1988, Chemiluminescence. Principles and Applications in Biology and Medicine (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)).
20　　　Furthermore, two types of liposomes may be targeted to the same cell type such that light is generated only when both are present.  For example, one liposome may carry luciferase, while the other carries luciferin.  The liposomes may carry targeting moieties, and the targeting moieties on
25 the two liposomes may be the same or different.  Viral proteins on infected cells can be used to identify infected tissues or organs.  Cells of the immune system can be localized using a single or multiple cell surface markers.
　　　　　The liposomes are preferably surface-coated, e.g., by
30 incorporation of phospholipid - polyethyleneglycol conjugates, to extend blood circulation time and allow for greater targeting via the bloodstream.  Liposomes of this type are well known.

35　　　　　5.　Cells.  Cells, both prokaryotic and eukaryotic, constitute another entity useful in the practice of the present invention.  Like particles, cells can be

loaded with relatively high concentrations of light-
generating moieties, but have the advantage that the light-
generating moieties can be provided by, for example, a
heterologous genetic construct used to transfect the cells.
5   In addition, cells can be selected that express "targeting
moieties", or molecules effective to target them to desired
locations within the subject.  Alternatively, the cells can
be transfected with a vector construct expressing an
appropriate targeting moiety.

10      The cell type used depends on the application.  For
example, as is detailed below, bacterial cells, such as
*Salmonella*, can be used to study the infective process, and
to evaluate the effects of drugs or therapeutic agents on the
infective process with a high level of temporal and spatial
15  resolution.

        Bacterial cells constitute effective entities.  For
example, they can be easily transfected to express a high
levels of a light-generating moiety, as well as high levels
of a targeting protein.  In addition, it is possible to
20  obtain *E. coli* libraries containing bacteria expressing
surface-bound antibodies which can be screened to identify a
colony expressing an antibody against a selected antigen
(Stratagene, La Jolla, CA).  Bacteria from this colony can
then be transformed with a second plasmid containing a gene
25  for a light-generating protein, and transformants can be
utilized in the methods of the present invention, as
described above, to localize the antigen in a mammalian host.

        Pathogenic bacteria can be conjugated to a light-
generating moiety and used in an animal model to follow the
30  infection process *in vivo* and to evaluate potential anti-
infective drugs, such as new antibiotics, for their efficacy
in inhibiting the infection.  An example of this application
is illustrated by experiments performed in support of the
present invention and detailed below.

35      Eukaryotic cells are also useful as entities in aspects
of the present invention.  Appropriate expression vectors,
containing desired regulatory elements, are commercially

available.  The vectors can be used to generate constructs
capable of expressing desired light-generating proteins in a
variety of eukaryotic cells, including primary culture cells,
somatic cells, lymphatic cells, etc.  The cells can be used
5  in transient expression studies, or, in the case of cell
lines, can be selected for stable transformants.

    Expression of the light-generating protein in
transformed cells can be regulated using any of a variety of
selected promoters.  For example, if the cells are to be used
10  as light-emitting entities targeted to a site in the subject
by an expressed ligand or receptor, a constitutively-active
promoter, such as the CMV or SV40 promoter may be used.
Cells transformed with such a construct can also be used to
assay for compounds that inhibit light generation, for
15  example, by killing the cells.

    Alternatively, the transformed cells may be administered
such they become uniformly distributed in the subject, and
express the light-generating protein only under certain
conditions, such as upon infection by a virus or stimulation
20  by a cytokine.  Promoters that respond to factors associated
with these and other stimuli are known in the art.  In a
related aspect, inducible promoters, such as the Tet system
(Gossen and Bujard, 1992, PNAS 89:5547-5551) can be used to
transiently activate expression of the light-generating
25  protein.

    For example, CD4+ lymphatic cells can be transformed
with a construct containing tat-responsive HIV LTR elements,
and used as an assay for infection by HIV (Israel and
Honigman, 1991, Gene 104:139-145).  Cells transformed with
30  such a construct can be introduced into SCID-hu mice (McCune,
et al., 1988, Science 241:1632-1639) and used as model for
human HIV infection and AIDS.

    Tumor cell lines transformed as above, for example, with
a constitutively-active promoter, may be used to monitor the
35  growth and metastasis of tumors.  Transformed tumor cells may
be injected into an animal model, allowed to form a tumor
mass, and the size and metastasis of the tumor mass monitored

during treatment with putative growth or metastasis
inhibitors.

   Tumor cells may also be generated from cells transformed
with constructs containing regulatable promoters, whose
5 activity is sensitive to various infective agents, or to
therapeutic compounds.


        6.    Cell Transformation.  Transformation methods
for both prokaryotic cells and eukaryotic cells are well
10 known in the art (Sambrook, et al., 1989, In Molecular
Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory
Press, Vol. 2).  Vectors containing the appropriate
regulatory elements and multiple cloning sites are widely
commercially available (e.g. Stratagene, La Jolla, CA,
15 Clontech, Palo Alto, CA).


IV.   Transgenic Animals Containing Genes Encoding Light-
      Generating Proteins
      In another aspect, the present invention includes
20 transgenic animals containing a heterologous gene construct
encoding a light-generating protein or complex of proteins.
The construct is driven by a selected promoter, and can
include, for example, various accessory proteins required for
the functional expression of the light-generating protein, as
25 well as selection markers and enhancer elements.
      Activation of the promoter results in increased
expression of the genes encoding the light-generating
molecules and accessory proteins.  Activation of the promoter
is achieved by the interaction of a selected biocompatible
30 entity, or parts of the entity, with the promoter elements.
If the activation occurs only in a part of the animal, only
cells in that part will express the light-generating protein.
      For example, an interferon-inducible promoter, such as
the promoter for 3'-5' poly-A synthetase, can be used to
35 detect the infection of transgenic cells by a number of
different RNA viruses.

In a related aspect, a promoter expressed in certain
disease states can be used to mark affected areas in a
transgenic animal, and expression of the light-generating
moiety can be used to monitor the effects of treatments for
5   the disease state.   For example, E-selection is expressed at
sites of inflammation *in vivo* (Pober and Cotran, 1991, *Lab.
Invest.* 64:301-305).   Accordingly, the E-selection promoter
can be isolated and used to drive the expression of a
luciferase gene.
10      It is also possible to use methods of the invention with
tissue-specific promoters.   This enables, for example, the
screening of compounds which are effective to inhibit
pathogenic processes resulting in the degeneration of a
particular organ or tissue in the body, and permits the
15  tracking of cells (*e.g.* neurons) in, for example, a
developing animal.
        Many promoters which are applicable for use with the
present invention are known in the art.   In addition, methods
are known for isolating promoters of cloned genes, using
20  information from the gene's cDNA to isolate promoter-
containing genomic DNA.


V.    Imaging of Light-Emitting Conjugates
        Light emitting conjugates that have localized to their
25  intended sites in a subject may be imaged in a number of
ways.   Guidelines for such imaging, as well as specific
examples, are described below.


        A.    Localization of Light-Emitting Conjugates
30      In the case of "targeted" entities, that is, entities
which contain a targeting moiety - a molecule or feature
designed to localize the entity within a subject or animal at
a particular site or sites, localization refers to a state
when an equilibrium between bound, "localized", and unbound,
35  "free" entities within a subject has been essentially
achieved.   The rate at which such an equilibrium is achieved
depends upon the route of administration.   For example, a

conjugate administered by intravenous injection to localize thrombi may achieve localization, or accumulation at the thrombi, within minutes of injection.  On the other hand, a conjugate administered orally to localize an infection in the

5 intestine may take hours to achieve localization.

Alternatively, localization may simply refer to the location of the entity within the subject or animal at selected time periods after the entity is administered.  For example, in experiments detailed herein, *Salmonella* are

10 administered (*e.g.*, orally) and their spread is followed as a function of time.  In this case, the entity can be "localized" immediately following the oral introduction, inasmuch as it marks the initial location of the administered bacteria, and its subsequent spread or recession (also

15 "localization") may be followed by imaging.

In a related aspect, localization of, for example, injected tumors cells expressing a light-generating moiety, may consist of the cells colonizing a site within the animal and forming a tumor mass.

20      By way of another example, localization is achieved when an entity becomes distributed following administration.  For example, in the case of a conjugate administered to measure the oxygen concentration in various organs throughout the subject or animal, the conjugate becomes "localized", or

25 informative, when it has achieved an essentially steady-state of distribution in the subject or animal.

In all of the above cases, a reasonable estimate of the time to achieve localization may be made by one skilled in the art.  Furthermore, the state of localization as a

30 function of time may be followed by imaging the light-emitting conjugate according to the methods of the invention.

B.   Photodetector Devices

An important aspect of the present invention is the

35 selection of a photodetector device with a high enough sensitivity to enable the imaging of faint light from within a mammal in a reasonable amount of time, preferably less than

about 30 minutes, and to use the signal from such a device to
construct an image.

     In cases where it is possible to use light-generating
moieties which are extremely bright, and/or to detect light-
5  emitting conjugates localized near the surface of the subject
or animal being imaged, a pair of "night-vision" goggles or a
standard high-sensitivity video camera, such as a Silicon
Intensified Tube (SIT) camera (e.g. Hamamatsu Photonic
Systems, Bridgewater, NJ), may be used.  More typically,
10 however, a more sensitive method of light detection is
required.

     In extremely low light levels, such as those encountered
in the practice of the present invention, the photon flux per
unit area becomes so low that the scene being imaged no
15 longer appears continuous.  Instead, it is represented by
individual photons which are both temporally and spatially
distinct form one another.  Viewed on a monitor, such an
image appears as scintillating points of light, each
representing a single detected photon.
20     By accumulating these detected photons in a digital
image processor over time, an image can be acquired and
constructed.  In contrast to conventional cameras where the
signal at each image point is assigned an intensity value, in
photon counting imaging the amplitude of the signal carries
25 no significance.  The objective is to simply detect the
presence of a signal (photon) and to count the occurrence of
the signal with respect to its position over time.

     At least two types of photodetector devices, described
below,  can detect individual photons and generate a signal
30 which can be analyzed by an image processor.


     1.    Reduced-Noise Photodetection Devices.  The
first class constitutes devices which achieve sensitivity by
reducing the background noise in the photon detector, as
35 opposed to amplifying the photon signal.  Noise is reduced
primarily by cooling the detector array.  The devices include
charge coupled device (CCD) cameras referred to as

"backthinned", cooled CCD cameras.  In the more sensitive
instruments, the cooling is achieved using, for example,
liquid nitrogen, which brings the temperature of the CCD
array to approximately -120°C.  The "backthinned" refers to
5  an ultra-thin backplate that reduces the path length that a
photon follows to be detected, thereby increasing the quantum
efficiency.  A particularly sensitive backthinned cryogenic
CCD camera is the "TECH 512", a series 200 camera available
from Photometrics, Ltd. (Tucson, AZ).
10

        2.    Photon Amplification Devices.  A second class
of sensitive photodetectors includes devices which amplify
photons before they hit the detection screen.  This class
includes CCD cameras with intensifiers, such as microchannel
15  intensifiers.  A microchannel intensifier typically contains
a metal array of channels perpendicular to and co-extensive
with the detection screen of the camera.  The microchannel
array is placed between the sample, subject, or animal to be
imaged, and the camera.  Most of the photons entering the
20  channels of the array contact a side of a channel before
exiting.  A voltage applied across the array results in the
release of many electrons from each photon collision.  The
electrons from such a collision exit their channel of origin
in a "shotgun" pattern, and are detected by the camera.
25      Even greater sensitivity can be achieved by placing
intensifying microchannel arrays in series, so that electrons
generated in the first stage in turn result in an amplified
signal of electrons at the second stage.  Increases in
sensitivity, however, are achieved at the expense of spatial
30  resolution, which decreases with each additional stage of
amplification.
        An exemplary microchannel intensifier-based single-
photon detection device is the C2400 series, available from
Hamamatsu.
35

        3.    Image Processors.  Signals generated by
photodetector devices which count photons need to be

- 28 -

processed by an image processor in order to construct an
image which can be, for example, displayed on a monitor or
printed on a video printer.  Such image processors are
typically sold as part of systems which include the sensitive
5 photon-counting cameras described above, and accordingly, are
available from the same sources (e.g. Photometrics, Ltd., and
Hamamatsu).  Image processors from other vendors can also be
used, but more effort is generally required to achieve a
functional system.

10      The image processors are usually connected to a personal
computer, such as an IBM-compatible PC or an Apple Macintosh
(Apple Computer, Cupertino, CA), which may or may not be
included as part of a purchased imaging system.  Once the
images are in the form of digital files, they can be
15 manipulated by a variety of image processing programs (such
as "ADOBE PHOTOSHOP", Adobe Systems, Adobe Systems, Mt. View,
CA) and printed.


         C.    Immobilizing Subject in Detection Field of Device
20            1.    Detection Field of Device.  The detection
field of the device is defined as the area from which
consistent measurements of photon emission can be obtained.
In the case of a camera using an optical lens, the detection
field is simply the field of view accorded to the camera by
25 the lens.  Similarly, if the photodetector device is a pair
of "night vision" goggles, the detection field is the field
of view of the goggles.
        Alternatively, the detection field may be a surface
defined by the ends of fiber-optic cables arranged in a
30 tightly-packed array.  The array is constructed to maximize
the area covered by the ends of the cables, as opposed to
void space between cables, and placed in close proximity to
the subject.  For instance, a clear material such as
plexiglass can be placed adjacent the subject, and the array
35 fastened adjacent the clear material, opposite from the
subject.

The fiber-optic cable ends opposite the array can be connected directly to the detection or intensifying device, such as the input end of a microchannel intensifier, eliminating the need for a lens.

5      An advantage of this method is that scattering and/or loss of photons is reduced by eliminating a large part of the air space between the subject and the detector, and/or by eliminating the lens. Even a high-transmission lens, such as the 60 mm AF Nikkor macro lens used in experiments performed

10 in support of the present invention, transmits only a fraction of the light reaching the front lens element.

With higher-intensity LGMs, photodiode arrays may be used to measure photon emission. A photodiode array can be incorporated into a relatively flexible sheet, enabling the

15 practitioner to partially "wrap" the array around the subject. This approach also minimizes photon loss, and in addition, provides a means of obtaining three-dimensional images of the bioluminescence.

Other approaches may be used to generate three-

20 dimensional images, including multiple detectors placed around the subject or a scanning detector or detectors.

It will be understood that the entire animal or subject need not necessarily be in the detection field of the photodetection device. For example, if one is measuring a

25 light-emitting conjugate known to be localized in a particular region of the subject, only light from that region, and a sufficient surrounding "dark" zone, need be measured to obtain the desired information.

30      2. Immobilizing the Subject. In those cases where it is desired to generate a two-dimensional or three-dimensional image of the subject, the subject may be immobilized in the detection field of the photodetection devices during the period that photon emission is being

35 measured. If the signal is sufficiently bright that an image can be constructed from photon emission measured in less than about 20 milliseconds, and the subject is not particularly

agitated, no special immobilization precautions may be
required, except to insure that the subject is in the field
of the detection device at the start of the measuring period.

    If, on the other hand, the photon emission measurement
5 takes longer than about 20 msec, and the subject is agitated,
precautions to insure immobilization of the subject during
photon emission measurement, commensurate with the degree of
agitation of the subject, need to be considered to preserve
the spatial information in the constructed image. For
10 example, in a case where the subject is a person and photon
emission measurement time is on the order of a few seconds,
the subject may simply be asked to remain as still as
possible during photon emission measurement (imaging). On
the other hand, if the subject is an animal, such as a mouse,
15 the subject can be immobilized using, for example, an
anesthetic or a mechanical restraining device.

    A variety of restraining devices may be constructed.
For example, a restraining device effective to immobilize a
mouse for tens of seconds to minutes may be built by
20 fastening a plexiglass sheet over a foam cushion. The
cushion has an indentation for the animal's head at one end.
The animal is placed under the plexiglass such that its head
is over the indentation, allowing it to breathe freely, yet
the movement of its body is constrained by the foam cushion.
25     In cases where it is desired to measure only the total
amount of light emanating from a subject or animal, the
subject does not necessarily need to be immobilized, even for
long periods of photon emission measurements. All that is
required is that the subject be confined to the detection
30 field of the photodetector during imaging. It will be
appreciated, however, that immobilizing the subject during
such measuring may improve the consistency of results
obtained, because the thickness of tissue through which
detected photons pass will be more uniform from animal to
35 animal.

D.    Further Considerations During Imaging

        1.    Fluorescent Light-Generating Moieties.  The
visualization of fluorescent light-generating moieties
requires an excitation light source, as well as a
5 photodetector.   Furthermore, it will be understood that the
excitation light source is turned on during the measuring of
photon emission from the light-generating moiety.

        Appropriate selection of a fluorophore, placement of the
light source and selection and placement of filters, all of
10 which facilitate the construction of an informative image,
are discussed above, in the section on fluorescent light-
generating moieties.


        2.    High-Resolution Imaging.  Photon scattering by
15 tissue limits the resolution that can be obtained by imaging
LGMs through a measurement of total photon emission.   It will
be understood that the present invention also includes
embodiments in which the light-generation of LGMs is
synchronized to an external source which can be focused at
20 selected points within the subject, but which does not
scatter significantly in tissue, allowing the construction of
higher-resolution images.   For example, a focused ultrasound
signal can be used to scan, in three dimensions, the subject
being imaged.   Light-generation from areas which are in the
25 focal point of the ultrasound can be resolved from other
photon emission by a characteristic oscillation imparted to
the light by the ultrasound (e.g. Houston and Moerner, U.S.
Patent No. 4,614,116, issued 30 September 1986.)


30    E.    Constructing an Image of Photon Emission
        In cases where, due to an exceptionally bright light-
generating moiety and/or localization of light-emitting
conjugates near the surface of the subject, a pair of "night-
vision" goggles or a high sensitivity video camera was used
35 to obtain an image, the image is simply viewed or displayed
on a video monitor.   If desired, the signal from a video
camera can be diverted through an image processor, which can

store individual video frames in memory for analysis or
printing, and/or can digitize the images for analysis and
printing on a computer.

Alternatively, if a photon counting approach is used,
5 the measurement of photon emission generates an array of
numbers, representing the number of photons detected at each
pixel location, in the image processor. These numbers are
used to generate an image, typically by normalizing the
photon counts (either to a fixed, pre-selected value, or to
10 the maximum number detected in any pixel) and converting the
normalized number to a brightness (greyscale) or to a color
(pseudocolor) that is displayed on a monitor. In a
pseudocolor representation, typical color assignments are as
follows. Pixels with zero photon counts are assigned black,
15 low counts blue, and increasing counts colors of increasing
wavelength, on up to red for the highest photon count values.
The location of colors on the monitor represents the
distribution of photon emission, and, accordingly, the
location of light-emitting conjugates.
20    In order to provide a frame of reference for the
conjugates, a greyscale image of the (still immobilized)
subject from which photon emission was measured is typically
constructed. Such an image may be constructed, for example,
by opening a door to the imaging chamber, or box, in dim room
25 light, and measuring reflected photons (typically for a
fraction of the time it takes to measure photon emission).
The greyscale image may be constructed either before
measuring photon emission, or after.

The image of photon emission is typically superimposed
30 on the greyscale image to produce a composite image of photon
emission in relation to the subject.

If it desired to follow the localization and/or the
signal from a light-emitting conjugate over time, for
example, to record the effects of a treatment on the
35 distribution and/or localization of a selected biocompatible
moiety, the measurement of photon emission, or imaging can be
repeated at selected time intervals to construct a series of

images.   The intervals can be as short as minutes, or as long
as days or weeks.


VI.   Analysis of Photon Emission Images
5      Images generated by methods and/or using compositions of
the present invention may be analyzed by a variety of
methods.   They range from a simple visual examination, mental
evaluation and/or printing of a hardcopy, to sophisticated
digital image analysis.   Interpretation of the information
10 obtained from an analysis depends on the phenomenon under
observation and the entity being used.
       The following experiments illustrate one application of
the present invention - tracking Salmonella infection in live
mice - and how images obtained using methods of the present
15 invention can be analyzed.


VII. Imaging of Luminescent Salmonella in Living Mice
       Experiments performed in support of the present
invention characterize the distribution of Salmonella
20 typhimurium infection in mice, the animal model of human
typhoid.   A mouse virulent Salmonella typhimurium strain,
SL1344 (Hoiseth and Stocker, 1981, Nature 291:238-239), a
non-invasive mutant of SL1344, BJ66 and a low virulence LT-2
strain of Salmonella, LB5000 were each marked with a plasmid
25 containing the lux operon, and used in experiments to
localize Salmonella infection in mice.


       A.    Constructions of Luminescent Salmonella
            1.    Salmonella Strains.   Three strains of
30 Salmonella typhimurium with differing virulence phenotypes,
defined by oral and intra-peritoneal inoculations into mice,
are selected for transformation.
       The most virulent phenotype used herein is SL1344, a
mouse strain originally obtained from a fatal infection of a
35 calf (Hoiseth and Stocker, 1981, Nature 291:238-239).
Following oral inoculations of mice with this strain,
bacteria are disseminated systematically via the lymphatic

system resulting in colonization of the liver, spleen and
bone marrow (Carter and Collins, 1974, *J. Exper. Med.*
<u>139</u>:1189-1203.; see also reviews by Finlay and Falkow, 1989,
*Mol. Microbiol.* <u>3</u>:1833-1841, and Hsu, 1989, *Microbiol. Rev.*
5 <u>53</u>:390-409.)

A non-invasive mutant of SL1344, BJ66, is also
evaluated.  Systemic infections in mice do not typically
result from an oral inoculation with BJ66, but do result from
intraperitoneal inoculations with this strain.

10      A low virulence LT-2 strain of *Salmonella*, LB5000, is
also examined.  LT-2 stains are laboratory strains known to
be of reduced or variable virulence for mice.  LB5000
contains multiple auxotrophic mutations, is streptomycin
resistant, and is cleared from mice following oral or
15 intraperitoneal inoculations.


        2.    Transformation of *Salmonella* Strains with the -
*lux* Operon.  The three strains are each transformed with a
plasmid encoding the *lux* operon, as detailed in Example 1.
20 The plasmid, obtained from the soil bacterium *Xenorhabdus
luminescens* (Frackman, *et al.*, 1990)  confers on *E coli* the
ability to emit photons through the expression of the two
subunits of the heterodimeric luciferase and three accessory
proteins, luxC, luxD and luxE.

25      Inclusion of luxC, luxD and luxE removes the necessity
of providing the fatty aldehyde substrate, luciferin, to the
luciferase-expressing cells.  Because supplying the substrate
to eukaryotic luciferase enzymes in an *in vivo* system such as
described herein may prove difficult, the entire *lux* operon
30 of *X. luminescens* is used.  The operon also encodes the
enzymes for the biosynthesis of the fatty aldehyde substrate.

        *X. luminescens* luciferase, an alpha-beta heterodimeric
mixed-function oxidase, catalyzes the oxidation of reduced
flavin and long-chain aldehyde to oxidized flavin and the
35 corresponding long-chain fatty acid.  A fatty acid reductase
complex is required for the generation and recycling of fatty

acid to aldehyde, and an NAD(P)H:flavin oxidoreductase
supplies the reduced flavin.

Optimal bioluminescence for *E. Coli* expressing the *lux*
genes of *X. luminescens* is 37°C (Szittner and Meighen, 1990,
5 *J. Biol. Chem.* 265:16581-16587, Xi, *et al.*, 1991, *J. Bact.*
173:1399-1405). In contrast, luciferases from eukaryotic and
other prokaryotic luminescent organisms typically have lower
temperature optima (Campbell, 1988, Chemiluminescence.
Principles and Applications in Biology and Medicine
10 (Chichester, England: Ellis Horwood Ltd. and VCH
Verlagsgesellschaft mbH)). The luciferase from *X.*
*luminescens*, therefore, is well-suited for use as a marker
for studies in animals.

The three strains are transformed by electroporation
15 with the plasmid pGSL1, which contains the entire *X.*
*luminescens* *lux* operon and confers resistance to ampicillin
and carbenicillin on the *Salmonella* (Frackman, *et al.*, 1990).
The *X. luminescens* *lux* operon contains the genes *luxA, luxB,*
*luxC, luxD* and *luxE* (Frackman, *et al.*, 1990). *LuxA* and *B*
20 encode the two subunits of the heterodimeric luciferase.
*luxC* and *D* encode the biosynthetic enzymes for the luciferase
substrate and *luxE* is a regulatory gene. Inclusion of the
genes for the biosynthesis of the substrate is a convenient
means of providing substrate to luciferase, in contrast to
25 supplying luciferin externally to the cells in culture or
treating animals with the substrate.


B.    Characterization of Transformed *Salmonella In Vitro*
1.    Adherence and Invasive Properties. The
30 adherence and invasive properties of the three *Salmonella*
strains containing the *lux* plasmid are compared in culture,
to each other, and to their non-luminescent parental strains
by the standard invasion assay as described by Finlay and
Falkow, 1989, *Mol. Microbiol.* 3:1833-1841., and detailed in
35 Example 2.

In this assay, adherent and intracellular bacteria are
quantified following incubation with an epithelial cell line

and peritoneal macrophages. The adherent and intracellular
bacteria are detected and quantified by both the emission of
photons from living cells, and colony forming units following
lysis and plating the cell lysates on carbenicillin-
5 containing plates.

The results of some of the assays are shown in Figures
2A through 2E and discussed in Example 8. The phenotypes of
the three strains transformed with the *lux* expressing plasmid
are not significantly altered in comparison to the parental
10 *Salmonella* strains. In addition, there is a good correlation
between the intensity of bioluminescence and the CFU from the
HEp-2 cells and macrophages. The results show that
luminescence, as an indicator of intracellular bacteria, is a
rapid method for assaying the invasive properties of bacteria
15 in culture.

BJ66 demonstrated reduced adherence to HEp-2 cells in
comparison to SL1344, however, adherence of the two strains
in primary cultures of murine peritoneal macrophages were
comparable.
20

2. <u>Light Emission</u>. To evaluate the oxygen
requirements of the system, 10 fold serial dilutions of
bacteria are placed in glass capillary tubes and imaged, as
detailed in Example 3.
25   Figure 3 shows an image generated in one such
experiment. Luminescence is only detected at the air-liquid
interface, even in the tubes with small numbers of bacteria
in air saturated medium (0.1 ml of air saturated buffer in 5
1 results in a final $O_2$ concentration of 5 nM).
30   From these results, it is apparent that oxygen is likely
a limiting factor for luminescence.


3. <u>Light Transmission Through Animal Tissue</u>. To
determine the degree to which light penetrates animal tissue,
35 light emitted from luminescent *Salmonella* and transmitted
through tissue is quantified using a scintillation counter,
with the fast coincidence detector turned off to detect

single photons.  The background due to dark current of the
photomultiplier tubes in this type of detection is
significant, limiting the assay to samples with relatively
strong photon emission.

5        Four tissue types of varying opacity are compared using
this approach: muscle from chicken breast, skin from chicken
breast, lamb kidney and renal medulla from lamb kidney.  The
number of photons that can be detected through tissue is
approximately ten fold less than the controls without tissue.

10

        4.    Characterization of *lux Salmonella in vivo*.
        a.    Oral Administration.  Oral inoculation is
natural route of infection of mice or humans with *Salmonella*
and results in a more protracted course of disease.  In order
15 to study the progression of the *Salmonella* infection
following this route of inoculation, two strains of mice are
infected with the three strains of *Salmonella*.  The results
obtained using the resistant animals are discussed under the
heading "Infection of Resistant Mice", below.

20       Balb/c mice are orally infected with suspensions of
virulent SL1344*lux*, non-invasive BJ66*lux* and low virulence
LB5000*lux Salmonella*, as described in Example 5.  Progression
of the infection is followed by external imaging (Materials
and Methods) over an 8 day period.

25       Representative images are shown in Figures 5A-F.  At 24
hours post inoculation (p.i.), the bioluminescent signal is
localized at a single focus in all infected animals (Figs.
5A, 5C and 5E).  Bioluminescence disappears in all animals
infected with the low virulence LB5000*lux* by 7 days p.i.
30 (Fig. 5B).  Animals infected with the virulent SL1344*lux*, on
the other hand, show virulent infection which often spreads
over much of the abdominal cavity (Fig. 5F), though the time
at which it begins to spread is highly variable from animal
to animal.  The infection by BJ66lux typically persists and
35 remains localized at a single site (Fig. 5D).

b.   I.P. Inoculation.  To assess whether or not
there is sufficient O$_2$ at the sites of *Salmonella* replication
for the oxidation of luciferin and subsequent luminescence
(Campbell, 1988, Chemiluminescence.  Principles and
5 Applications in Biology and Medicine (Chichester, England:
Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)), photon
emission is measured from the tissues of a respiring animal.
Luminescent SL1344*lux* and LB5000*lux* are inoculated into the
peritoneal cavities of two groups of Balb/c mice.  32 hours
10 post inoculation (p.i.), the transmitted photons are imaged
(Figure 6).

In the mice infected with SL1344*lux* (left part of
figure), transmitted photons are evident over a large
surface, with foci of varying intensities visible.  These
15 images are indicative of a disseminated infection, and are
consistent with widespread colonization of the viscera,
possibly including the liver and mesenteric lymph nodes.  In
contrast, the distributions of transmitted photons from
animals infected with the LB5000*lux* strain is very limited,
20 indicating a limited infection.

The LB5000*lux*-infected mice remained healthy for several
weeks p.i., while the SL1344*lux*-infected mice were nearly
moribund and euthanized at 4 days p.i.

These experiments indicate that the level of O$_2$ in the
25 blood and or tissues is adequate for bioluminescence of *lux*
luciferase expressed by *Salmonella*.  Furthermore, the
experiments are consistent with the invasive nature of the
virulent strain SL1344 in comparison to the reduced virulent
laboratory strain LB5000.
30

c.   Infection of Resistant Mice.  Mice which are
heterozygous at the *Ity* locus (*Ity*^r/s^) are resistant to
systemic infections by *S. typhimurium* (Plant and Glynn, 1976,
*J. Infect. Dis.* 133:72-78).  This locus, also called *Bcg*
35 (Gros, *et al.*, 1981, *J Immunol.* 127:2417-2421) or *Lsh*
(Bradley, 1977, *Clin. and Exper. Immunol.* 30:130-140),
regulates the pathogenic processes of certain intracellular

pathogens, such as *Mycobacterium lepraemurium* (Forget, *et al.*, 1981, *Infect. Immunol.* 32:42-47), *M. Bovis* (Skamene, *et al.*, 1984, *Immunogenet.* 19:117-120, Skamene and Pietrangeli, 1991, *Nature* 297:506-509) and *M. intracelluare* (Goto, *et al.*,
5 1989, *Immunogenetics* 30:218-221). An analogous genetic control of resistance and susceptibility to intracellular pathogens appears to be in humans as well (*M. tuberculosis* (Stead, 1992, *Annals of Intern. Med.* 116:937-941, Stead, *et al.*, *et al.*, 1990, *New Eng. J. Med.* 322:422-427) and *M.*
10 *leprae*).

The *Ity* locus is located on mouse chromosome 1 with two allelic forms, *Ity$^r$* (resistant, dominant) and *Ity$^s$* (sensitive, recessive). The gene encoded at the *Ity* locus apparently affects the ability of macrophages to disrupt the
15 internalized pathogens (reviewed by Blackwell, *et al.*, 1991, *Immunol. Lett.* 30:241-248 (1991); see also Skamene, *et al.*, 1984, *Immunogenet.* 19:117-120, Skamene and Pietrangeli, 1991, *Nature* 297:506-509) which in turn, affects the down stream function of the proposed macrophage-mediated transport of
20 pathogens to other sites within the infected host. Balb\c mice are *Ity$^{s/s}$* and 129 mice are *Ity$^{r/r.}$* The heterozygous Balb\c x 129 mice (*Ity$^{r/s}$*) are used in experiments detailed herein.

Resistant 129xBalb/c (*Ity $^{r/s}$*) viable mice are infected
25 by intragastric inoculation of $1 \times 10^7$ SL1344*lux Salmonella* as detailed in Example 7. The animals are imaged daily for 8 days post injection (d.p.i.).

Results are shown in Figures 7A (day 1) and 7B (day 8). The luminescence, detected by external imaging, is apparent
30 at 24 h p.i., and appeared to localized to a single site in all animals. The luminescent signal is present throughout the study period (up to 8 days p.i.). The intensity of the luminescence and the location of the luminescent source is somewhat variable over time within a mouse and also from
35 mouse to mouse. The luminescent tissue in all infected animals is the cecum (see below) and the variability in localization, and possibly intensity, is most likely due fact

that internal organs of rodents are not tightly fixed in
position.

The apparent limited infection observed in these animals
supports the interpretation that the *Ity* restriction blocks
5  macrophage transport.  The persistence of this infection for
10 days, however, suggests that there is adherence to the
intestine mucosa and prolonged shedding of bacteria in the
feces of these animals, as evidenced by luminescent fecal
pellets.  These results indicate that the luminescent
10 phenotype of the *Salmonella* in vivo is retained over an 8 day
duration in *Ity* restricted animals and that localization is
possible following an oral inoculation.


          d.    Internal Imaging Following Oral Inoculation.
15 In order to further localize the luminescent signal in the
abdominal cavity, infected mice are imaged following .
laparotomy (Example 8).  The predominant disease
manifestation in all of the animals infected by the oral
route is an enlarged cecum (Figs. 8A-C).  The "external"
20 image (Fig. 8A) illustrates a focal luminescence, which is
revealed in the post-laparotomy image (Fig. 8B) to be the
cecum.

Injection of air into the intestine confirms the
presence of bacteria in other regions of the digestive tract.
25 Bacteria in the colon and rectum are likely expressing
luciferase, but low oxygen concentrations are likely limiting
light emission from these sites.

The images obtained from oral inoculation studies
indicate that the luminescent signal, at 2 days p.i. and at 7
30 days p.i., localizes almost entirely to the cecum in each of
the animals (Popesko, *et al.*, 1990, A Colour Atlas of Anatomy
of Small Laboratory Animals Vol. Two: Rat Mouse Hamster
(London England: Wolfe)) except those infected with
LB5000*lux*.  Luminescence is also apparent in the colon in
35 some animals.  By 7 days p.i., no luminescence is detectable
in the LB5000*lux*-infected animals.  The CFU present in the
organs of these mice are determined at 2 and 5 d p.i.

In animals infected intragastrically with the invasive
strain, SL1344*lux*, the luminescence in the cecum appears
early and precedes a systemic infection.  In contrast,
infections with the non-invasive BJ66*lux* strain result in a
5 persistent luminescence from the cecum that remains, in some
animals, for the entire course of the study (8 days).  By 8
days p.i., luminescence is detected over much of the
abdominal surface, resembling the distribution of photons
following an i.p. inoculation, in the SL1344*lux* infected
10 mice.

Infections with SL1344*lux* appear to become systemic, as
predicted, with progressively more photons being emitted from
an  increasing surface area.  Luminescence appears to
localize over the abdomen in infections with all strains with
15 little detectable luminescence from outside this area.  A
large number of transmitted photons are localized as a single
focus over the abdomen suggesting that even though the
infection may be systemic, the greatest amount of replication
may be in areas surrounding the intestine.

20 Localization of the luminescence over the cecum
indicates that not only are there large numbers of organisms
in this region of the intestine, but also suggests that the
*Salmonella* associate with cells of the mucosa such that they
can obtain sufficient oxygen for luminescence.  Emission of
25 photons from luciferase is oxygen dependent and the expected
oxygen levels in the lumen of the cecum, or intestine in
general, are below the levels required for luminescence.  The
luciferase reaction is not expected to be functional in the
intestine unless the bacteria can obtain oxygen from cells of
30 the intestinal epithelium.

Thus, the systemic infection seems to be related to the
invasive phenotype and not to simply adherence to epithelial
cells of the intestine.  These experiments implicate the
cecum in some role in the pathogenic process either in the
35 carrier state or as a site of dissemination.

Monitoring the progression of infections to different
tissues may greatly enhance the ability to understand these

steps in the pathogenic process, and enable the screening for
compounds effective to inhibit the pathogen at selected
steps.


5          e.   Internal Imaging Following I.P. Inoculation.
Mice infected intraperitoneally with SL1344lux are imaged
before and after laparotomy (Example 9). The results are
shown in Fig. 9. The images demonstrate luminescence over a
majority of the abdomen with multiple foci of transmitted
10 photons. The cecum does not appear to contain luminescent
*Salmonella*. The results from these experiments indicate that
all strains of *Salmonella* have sufficient $O_2$ to be luminescent
in the early phases of infection. However, entry of
*Salmonella* into cells of the mucosa and subsequent systemic
15 infection is likely limited to strains with the invasive
phenotype, since systemic infections at later time points are
only apparent in SL1344*lux*-infected mice.


          f.   Effects of Ciprofloxacin on *Salmonella*
20 Infection. Experiments, detailed in Example 10, are
performed to demonstrate that non-invasive imaging is useful
for following the response of an infection to drugs. Mice
are orally inoculated with SL1344*lux* and treated with 100 mg
of ciprofloxacin, an antibiotic effective against *Salmonella*
25 infections. The mice are imaged at selected time periods
following treatment, and the extent of infection is
quantitated by measuring photon emission. Photon emission in
treated mice is compared to values before the initiation of
treatment, and to values from control mice that had been
30 infected, but not treated. Results from one such experiment
are shown in Figures 10A-E and discussed in Example 10.
Infection is significantly reduced in mice treated with the
antibiotic, compared both to the levels of pathogen at time
zero in treated animals, and to levels of pathogen in control
35 animals throughout the treatment period.

        g.    Effects of Carbenenicillin Selection.
Ducluzeau, *et al.*, 1970, *Zeut. Bakt.* 5313:533-548.,
demonstrated that treatment of animals with antibiotics
facilitated colonization of the cecum with *Salmonella*.  The
5 mice in the present experiments are maintained on an
antibiotic regime of intramuscular injections of
carbenicillin for the purpose of selecting the Amp^r *Salmonella*
containing the luciferase clone.  This treatment may alter
the course of the gastrointestinal infection, but the
10 observation that *Salmonella* can associate with the cells
lining the cecum indicates that oxygen is available for
luminescence.  This observation is notable, since the lumen
of the cecum is commonly thought to be an anaerobic
environment.
15

VIII. Applications

     The bioluminescence technology is broadly applicable to
a variety of hostpathogen systems and may also enable
temporal and spatial evaluation of other biological events,
20 as for example tumor progression and gene expression in
living mammals, and have application in pharmaceutical
development and screening.  Widespread use of *in vivo* imaging
of pathogens may reduce the numbers of animals and time
needed for experiments pertaining to pathogenesis and/or the
25 real-time study antimicrobial agents.  Furthermore,
bioluminescent organisms may be useful as biosensors in the
living animal, much as luminescent bacteria are used in
environmental analyses.  Korpela *et al.*, for example,
demonstrate that the limited oxygen supply in the lumen of
30 the G.I. tract restricted bioluminescence to sites in which
oxygen is accessible to the *Salmonella*, perhaps directly from
epithelial or other cell types.  Korpela, *et al.*, 1989, *J.*
*Biolum. Chemilum.* 4:551-554.  This oxygen requirement may
find utility as an indicator of intimate cell-cell
35 interactions, or as a biosensor for studying oxygen
concentrations at various sites in living animals.  In the
following, several exemplary applications of this technology

are described for the purpose of illustration, but are in no
way intended to limit the present invention.


   A.   Determination of Oxygen Levels
5      The oxygen requirement for luminescence of luciferase
evidenced in the experiments summarized above indicates that
the present invention may be applicable as a method of
determining spatial gradients of oxygen concentration in a
subject.  Luminescent bacteria have been used to measure
10 oxygen levels in the range of 10 - 1 mM.  The studies predict
that 0.1 nM is the lower limit of detection (Campbell, 1988,
Chemiluminescence.  Principles and Applications in Biology
and Medicine (Chichester, England: Ellis Horwood Ltd. and VCH
Verlagsgesellschaft mbH)).  The imaging methods described
15 herein may be used for studying oxygen levels at various
sites in living animals.  For example, microorganisms that
have been engineered to emit light in an $O_2$ or $Ca^{2+}$ -dependent
manner could be used as biosensors in a subject, much like
luminescent bacteria are used in environmental analyses
20 (Guzzo, et al., 1992, Tox. Lett. 64/65:687-693, Korpela, et
al., 1989, J. Biolum. Chemilum. 4:551-554, Jassim, et al.,
1990, J. Biolum. Chemilum. 5:115-122).  The dynamic range of
luminescence with respect to $O_2$ concentration is much broader
and reaches lower $O_2$ concentrations than $O_2$ probes (Campbell,
25 1988, Chemiluminescence.  Principles and Applications in
Biology and Medicine (Chichester, England: Ellis Horwood Ltd.
and VCH Verlagsgesellschaft mbH)).  Moreover, light emission
in proportion to $O_2$ concentration is linear over a range of 30
nM to 8 mM, and 9 mM $O_2$ is required for 1/2 maximal
30 luminescence.


   B.   Localization of Tumor Cells
   The growth and metastatic spread of tumors in a subject
may be monitored using methods and compositions of the
35 present invention.  In particular, in cases where an
individual is diagnosed with a primary tumor, LECs directed
against the cells of the tumor can be used to both define the

- 45 -

boundaries of the tumor, and to determine whether cells from the primary tumor mass have migrated and colonized distal sites.

For example, LECs, such as liposomes containing

5 antibodies directed against tumor antigens and loaded with LGMs, can be administered to a subject, allowed to bind to tumor cells in the subject, imaged, and the areas of photon emission can be correlated with areas of tumor cells.

In a related aspect, images utilizing tumor-localizing

10 LECs, such as those described above, may be generated at selected time intervals to monitor tumor growth, progression and metastasis in a subject over time. Such monitoring may be useful to record results of anti-tumor therapy, or as part of a screen of putative therapeutic compounds useful in

15 inhibiting tumor growth or metastasis.

Alternatively, tumor cells can be transformed with a luciferase construct under the control of a constitutively-active promoter, and used to induce luminescent tumors in animal models, as described above. Such animal models can be

20 used for evaluating the effects of putative anti-tumor compounds.


C. Localization of Inflammation

In an analogous manner to that described above,

25 compositions and methods of the present invention may be used to localize sites of inflammation, monitor inflammation over time, and/or screen for effective anti-inflammatory compounds. Molecules useful for targeting to sites of inflammation include the ELAN family of proteins, which bind

30 to selections. An ELAN molecule can be incorporated as a targeting moiety on an entity of the present invention, and used to target inflammation sites.

Alternatively, an animal model for the study of putative anti-inflammatory substances can be made by making the animal

35 transgenic for luciferase under the control of the E-selection promoter. Since E-selection is expressed at sites

of inflammation, transgenic cells at sites of inflammation
would express luciferase.

The system can be used to screen for anti-inflammatory
substances.  Inflammatory stimuli can be administered to
5  control and experimental animals, and the effects of putative
anti-inflammatory compounds evaluated by their effects on
induced luminescence in treated animals relative to control
animals.


10       D.    Localization of Infection
As illustrated in experiments performed in support of
the present invention and summarized above, LGCs may be
effectively used to follow the course of infection of a
subject by a pathogen.  In experiments detailed herein, the
15 LGCs are pathogenic cells (*Salmonella*) transformed to express
luciferase.  Such a system is ideally-suited to the study of
infection, and the subsequent spread of infection, in animal
models of human diseases.  It provides the ability to monitor
the progression of an infectious disease using sites of
20 infection and disease progression rather than traditional
systemic symptoms, such as fever, swelling, etc. in studies
of pathogenesis.

Use of an external imaging method to monitor the
efficacy of anti-infectives permits temporal and spatial
25 evaluations in individual living animals, thereby reducing
the number of animals needed  for experiments pertaining to
pathogenesis and/or the study anti-infective agents.


The following examples illustrate, but in no way are
30 intended to limit the present invention.


### MATERIALS AND METHODS

A.    Cells

*Salmonella* strains SL1344 and LB5000 were obtained from
35 B.A.D. Stocker (Stanford University; Hoiseth and Stocker,
1981, *Nature* 291:238-239).  *Salmonella* strain BJ66 was
obtained from B.D. Jones (Stanford University).

- 47 -

HEp-2 cells were obtained from the American Type Culture
Collection (ATCC; 12301 Parklawn Dr., Rockville MD;
Accession number CCL-23).

Murine peritoneal macrophages were obtained by
5 peritoneal lavage of euthanized Balb/c mice with 7 ml of
growth medium (Maximow and Bloom, 1931, Textbook of
Histology, Saunders, Philadelphia.)

B.    Static Cultures
10    Low oxygen (static) cultures were prepared by
inoculating 3 ml of LB Broth containing 100 mg/ml of
carbenicillin with 6 $\mu$l of a bacterial suspension from a
stationary phase culture, and growing the bacteria at 37°C
overnight in a stationary 7 ml culture tube.
15

C.    Mice
Balb/c ($Ity^{s/s}$) mice were obtained from the Department of
Oncology, Stanford University.  129xBalb/c ($Ity^{r/s}$) mice were
obtained from the Stanford Transgenic Animal Facility
20 (Stanford, CA).  All animals were housed under identical
conditions of photo period, feeding regime and temperature in
the Stanford University Research Animal Facility (Stanford,
CA).

Anesthesia was performed by injecting the animals
25 intraperitoneally (i.p.) with 33 $\mu$g/kg body weight nembutal.
Euthanasia was performed by asphyxiation in $CO_2$ or
cervical dislocation, following protocols recommended by the
Stanford University Research Animal Facility.  Cervical
dislocation was used in experiments in which results may have
30 been affected by physiological changes due to asphyxia.

Mice infected with lux-transformed Salmonella were given
daily intramuscular (i.m.) injections of carbenicillin (125
mg per kg body weight) to maintain selective pressure on the
luminescent Salmonella for retention of the $Amp^r$ plasmid
35 containing the lux operon.

D.    Imaging

Animals or objects to be imaged were immobilized in a
light-tight box containing a door and a charge-coupled device
(CCD) camera with a two stage microchannel intensifier head
5 (model C2400-40, Hamamatsu).  The camera was attached, via
cables leading out of the box, to an "ARGUS 50" image
processor (Hamamatsu).

The ICCD system described above is capable of detecting
single photons once a threshold of 10-30 photons is achieved.
10 The signal to noise ratio of the system ranged from 2:1 to
$1x10^4$:1, depending on signal intensity.

Grey-scale images were obtained by opening the light box
door in dim room light and integrating for 8 - 64 frames.
The gain for the gray scale images was set to optimize the
15 image - typically at 3000 volts on a scale of 0 to 10,000
volts.

Bioluminescence data were obtained in absence of
external illumination.  Exposure settings were as follows:
the black level was set automatically by the camera/image
20 processor, the gain was adjusted automatically by the
intensifier controller, and the f-stop was set at 2.8.  A 60
mm "AF NIKKOR" macro lens was used (Nikon Inc., Melville,
NY).

Bioluminescence images were generated by integrating
25 photons for a selected period of time, typically 5 minutes.
Data are presented at the lowest bit range setting of 0-3
bits per pixel for all animals.  For images of other objects,
i.e. 24 well plates, where the resolution of the
bioluminescent signals was not possible at a bit range of 0-
30 3, the range was increased to a setting that permitted
localization of bioluminescent signals, typically 1-7.
Objects were imaged for shorter periods of time when
additional information could not be obtained by imaging for
five minutes.
35     External imaging refers to non-invasive imaging of
animals.  Internal imaging refers to imaging after a partial
dissection of the animals, typically a laparotomy.  Internal

- 49 -

imaging is performed in selected animals to confirm the
sources of photon emission localized by external imaging.

The bioluminescence image data are presented as a
pseudo-color luminescence image representing the intensity of
5  the detected photons.  Six levels of intensity are typically
used, ranging from blue (low intensity) to red (higher
intensity).

To generate the figures presented herein, greyscale and
bioluminescence images were superimposed, using the image
10  processor, to form a composite image providing a spatial
frame of reference.

The composite image was displayed on an RGB CRT (red,
green, blue; cathode ray tube) monitor, and the monitor was
photographed to produce hardcopies.  Hardcopies were also
15  generated by saving the image processor image as a digital
file, transferring the file to a computer, and printing it on
a color printer attached to the computer.  Alternatively,
hardcopies may be generated by printing the video signal
directly using a video printer.

20

## EXAMPLE 1

### TRANSFORMATION OF *SALMONELLA* WITH PCGLS1 *LUX* PLASMID

*Salmonella* strains SL1344, BJ66 and LB5000 were
transformed with pCGLS1, a pUC18-based vector encoding the
25  *lux* operon from *Xenorhabdus luminescens* (Frackman, *et al.*,
1990).


A.   pCGLS1 plasmid

A schematic of the pCGLS1 plasmid is shown in Figures
30  1A, 1B and 1C.  The plasmid was constructed by cloning an ~11
kb region encoding the *lux* genes from the soil bacterium
*Xenorhabdus luminescens* (Fig. 1A; Frackman, *et al.*, 1990)
into the *Bam* HI site (Fig. 1B) of pUC18 (Fig. 1C; Clontech,
Palo Alto, CA).  The construction of the vector is described
35  by Frackman, *et al.*, (1990).

Restriction enzyme sites in Figure 1A are represented as
follows: Bs, *Bst* EII; C, *Cla* I; E, *Eco* RI; H, *Hind* III; M,

*Mlu* I; S, *Sca* I; X, *Xba* I; B/Sa, *Bam* HI and *Sau* 3A junction.
A sequence included in the multiple cloning site (MCS) is
provided in Figure 1B, with the *Bam* HI site indicated in bold
type.

5       A graphical representation of a pUC18 vector with no
insert is shown in Figure 1C. Labeled elements include an
ampicillin resistance gene (Ap), a *lac* Z gene (*lac* Z) and an
*E. coli* origin of replication (*Ori*). The unmodified pUC18
vector is approximately 2.7 kb in size.

10

        B.    Transformation of *Salmonella*
        Electrocompetent cells from *Salmonella* strains SL1344,
BJ66 and LB5000 were made using standard methods (Sambrook,
*et al.*, 1989, In <u>Molecular Cloning: A Laboratory Manual</u>, Cold

15 Spring Harbor Laboratory Press, Vol. 2) and stored at -80°C
until just prior to use. Electroporation was performed as
follows: 1 $\mu$l of the plasmid (0.2 $\mu$g/ml) was added to 40 $\mu$l
of ice-cold electrocompetent cells suspended in 10% glycerol.
The suspension was mixed gently for one minute, placed in a 1

20 mm gap electroporation cuvette and electroporated using a
Bio-Rad Gene-Pulser (Bio-Rad Laboratories, Hercules, CA).
The settings were 2.5 kvolts, 400 ohms and 25 $\mu$farads.
        Following a one hour agitated incubation in Luria
Bertini (LB) broth at 37°C, the cells were plated on (LB)

25 Agar containing 100 $\mu$g/ml carbenicillin and allowed to grow
overnight.
        To maximize the bioluminescence of the labelled
*Salmonella*, the *lux* operon was maintained on a high-copy-
number plasmid and not integrated as a single copy gene.

30 However, plasmids are subject to modification by the
bacterial cell especially in *recA* strains, such as SL1344 and
BJ66 used in this study. The *recA* locus encodes a
recombinase that may delete regions of the plasmid containing
the *lux* operon and the $\beta$-lactamase. Therefore, *Salmonella*

35 recovered from cells in culture were plated both in the
presence or absence of carbenicillin, and were imaged to
determine the frequency at which bioluminescence was lost.

All colonies recovered from gentamicin-treated, lysed HEp-2
cells and macrophages were ampicillin resistant (Amp$^r$) and
bioluminescent. Therefore, *lux* genes appeared not to be lost
during co-culture with mammalian cells.

5      Colonies were assayed for luminescence by visual
inspection in a dark room. Five transformants were
identified as having high levels of luminescence. Three of
these, one each from the SL1344, BJ66 and LB5000 strains,
were selected for subsequent experiments. They were termed
10   SL1344*lux*, BJ66*lux* and LB5000*lux*, respectively.


## EXAMPLE 2
### INVASIVE POTENTIAL OF NORMAL AND TRANSFORMED *SALMONELLA*
The invasive potential of six strains of *Salmonella*
15   (SL1344*lux*, LB5000*lux*, BJ66*lux*, SL1344, LB5000 and BJ66) was
determined using two types of bacterial adherence and entry
assays. Colony-forming units (CFU) assays were performed
essentially as previously described (Finlay and Falkow, 1989,
*Mol. Microbiol.* 3:1833-1841) with modifications (Lee, *et al.*,
20   1990, *PNAS* 87:4304-4308). Bioluminescence assays were
performed essentially like the CFU assays, except that the
number of cells was quantitated using bioluminescence, as
opposed to CFUs.
       Briefly, HEp-2 cells and primary murine peritoneal
25   macrophages were seeded into 24-well tissue culture dishes at
1 × 10$^5$ cells per well in RPMI (Gibco/BRL, Grand Island, NY)
supplemented with 20 mM glutamine (Gibco/BRL) and 5% fetal
calf serum (Hyclone, Logan, UT). Twenty four hours (HEp-2)
or seven days (macrophages) after cell seeding, bacteria from
30   static cultures (see "Materials and Methods", above) were
inoculated at 1 × 10$^6$ (multiplicity of infection (m.o.i.) of
10) or 1x10$^7$ (m.o.i. of 100, columns on right in Figs. 2B-E)
organisms per well and centrifuged onto the cell monolayer
for 5 minutes at 1000 rpm (185 × g) in a Beckman clinical
35   centrifuge (Beckman Instruments, Columbia, MD). The medium
was replaced with RPMI medium (Gibco/BRL) either with (entry
assay) or without (adherence assay) gentamicin (100 mg/ml).

The co-cultures were incubated for a total of 3.5 hours at 35°C in 5% $CO_2$.

Gentamicin in the incubation medium kills bacteria that had not been internalized by the HEp-2 cells, including those
5  adhering to the surfaces of the HEp-2 cells. Accordingly, the signal in adherence assays (without gentamicin) represent both adherent and internalized bacteria, whereas the signal in entry assays (with gentamicin) represent only internalized bacteria.

10     Adherence and entry were assayed by imaging luminescent bacterial cells at three timepoints - 1.5, 3.0 and 3.5 hours post inoculation. Prior to imaging at the first timepoint, the cell monolayer was washed three times with phosphate-buffered saline (PBS) to remove unattached bacteria and a
15  fresh aliquot of RPMI medium was added. Luminescence was recorded using a 30 second exposure. Images at the second and third timepoints were obtained using a similar exposure, but without first washing the cells.

Data recorded at the last timepoint, displayed as
20  pseudocolor luminescence images superimposed over gray scale images of the culture dish wells, are shown in Figure 2A. The cell types, *Salmonella* strains, and usage of gentamicin are indicated in the Figure. The data are also summarized as relative intensity of photon counts in the graphs in Figures
25  2B and 2D.

Following imaging at the 3.5 hour timepoint, the tissue culture cells were washed three times with PBS and lysed with 0.2% "TRITON X-100" in PBS. Adherent and/or intracellular bacteria, released by lysis, were plated on LB- or LB-
30  carbenicillin agar plates and incubated for 18 h at 35°C. The number of bacteria released from each well was determined by counting the number of colony forming units (CFU, Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833-1841., Lee, *et al.*, 1990, *PNAS* 87:4304-4308). These data are represented as the
35  total bacterial colonies per ml recovered from co-culture after incubation for 3.5 h with or without gentamicin, and are summarized in the graphs in Figures 2C and 2E.

- 53 -

Data from both the bioluminescence and CFU assays indicate that (i) *Salmonella* transformed with the *lux* genes have an infective potential similar to that of the parent lines, and (ii) luminescence detection and CFU determination
5  yield comparable estimates for the invasive potential of the two *Salmonella* strains in HEp-2 cells and macrophages. The ratio of bioluminescence to CFU was lower in macrophage cultures, possibly due to the subcellular compartment in which the *Salmonella* enter macrophages.
10

### EXAMPLE 3

#### IN VITRO LUMINESCENCE OF TRANSFORMED SALMONELLA

10 $\mu$l of four 10-fold serial dilutions (ranging from $10^6$ cells to $10^3$ cells per ml) of LB5000*lux Salmonella* were placed
15  in four 100 $\mu$l glass capillary tubes (Clay-Adams div. of Becton Dickinson, Parsippany, NJ). The bacterial suspensions formed columns of fluid in the tubes, with pockets of air at both ends. One end of each tube was sealed with critoseal (Clay-Adams). The medium in which dilutions were made was
20  saturated with $O_2$ through exposure to air.

The tubes were wrapped with clear plastic wrap and luminescence was determined by imaging for 30 seconds as described above. An exemplary image is shown in Figure 3A. Four tubes are pictured. They contained (from top to bottom)
25  $10^6$, $10^5$, $10^4$ and $10^3$ *Salmonella* cells/ml ($10^4$, $10^3$, $10^2$ and 10 cells/tube). Luminescence could be detected in suspensions containing as few as $10^4$ cells/ml (100 cells). The luminescence is confined, however, to air/liquid interfaces, suggesting that the luminescence reaction requires relatively
30  high levels of oxygen. Since many of the cells are presumably in the fluid column and not at the air/fluid interfaces, the data suggest that the luminescence in the capillary tubes shown in Fig. 3A arises from considerably fewer than the total number of cells in each tube.
35

EXAMPLE 4

*IN VITRO* DETECTION OF LUMINESCENCE THROUGH ANIMAL TISSUE

Micro test-tubes, constructed from glass capillary
tubing with an internal diameter of 3.5 mm, containing serial

5 dilutions of LB5000*lux Salmonella* were prepared essentially
as described in Example 3, above.  In the present example,
however, the bacterial suspensions contacted the sealed end
of the tube and were exposed to air only at the upper end.
The tubes were placed in a translucent plastic scintillation

10 vial and surrounded by one of the following animal tissues:
chicken breast muscle, chicken skin, lamb kidney or lamb
renal medulla.  All tissues were obtained from the meat
department of a local supermarket (Safeway, Mountain View,
CA).

15     A diagram of a vial containing a capillary tube
surrounded by tissue is shown in Figure 4.  The vial 1 is
approximately 1.4 cm in diameter and includes a cap 2.  The
vial is coated with an opaque material (*i.e.* black tape)
along its upper portion 3.  Animal tissue 4 is placed in the

20 vial such that it extends from the bottom of the vial to just
above the bottom edge of the opaque coating 3.  The micro
test-tube 5 is sealed at the bottom by a plug 7 (*i.e.* a
crytoseal plug), and is centered radially in the vial, with
the plugged end of the tube touching or in close proximity to

25 the bottom of the vial.  The bacterial suspension 6 extends
approximately 1 cm upward from the bottom of the tube.

Photons emitted from vials with and without tissue, and
with and without bacteria, were counted using a liquid
scintillation counter (model 1219 Rackbeta, LKB/Wallac,

30 Gaithersburg, MD) with the fast coincidence discriminator
disabled.

Controls without tissue were assayed by placing the
bacterial suspension directly in the scintillation vial.  All
experiments were performed in triplicate.

35     In each experiment, the vials were counted two to three
times, rotating the vial 90° between each count, to control

for effects of possible tissue thickness inconsistency. No
significant differences were detected.

The results are summarized in Table 1, below.

5

TABLE 1

| TRANSMISSION OF PHOTONS THROUGH TISSUE | | | | |
|---|---|---|---|---|
| Sample | Chicken skin | Chicken muscle | Lamb kidney | Lamb medulla |
| Vial alone | $2.1 \times 10^4$ | $1.3 \times 10^4$ | $1.0 \times 10^4$ | $1.0 \times 10^4$ |
| Tissue alone | N.D. | $1.5 \times 10^4$ | $9.4 \times 10^3$ | $8.5 \times 10^3$ |
| Tissue and LB5000lux* | $2.7 \times 10^5$ | $2.3 \times 10^5$ | $1.6 \times 10^4$ | $1.5 \times 10^5$ |
| LB5000lux* alone | $2.0 \times 10^6$ | $1.7 \times 10^6$ | $4.8 \times 10^6$ | $4.8 \times 10^6$ |

10

15

Counts are averages of triplicate measurements, tissue path length
was 1 cm. * - $1 \times 10^7$ cells.

20      The signal for $1 \times 10^3$ LB5000*lux* in kidney tissue was at or
near background levels using the photomultiplier tubes (PMT)
in the scintillation counter. The background in this type of
detection is due to the dark current of the PMT and limits
the studies to analysis of rather intense signals.

25      Bioluminescence from approximately $1 \times 10^7$ LB5000*lux* was
detectable through 0.5 cm of avian muscle, skin ovine renal
medulla and ovine kidney. These results indicate that
bioluminescence from the labeled *Salmonella* was detectable
through animal tissues of variable opacity. Since oxygen was
likely limited in the capillary tubes (as demonstrated in
30      Fig. 3A), it is likely that fewer numbers of bioluminescent
*Salmonella* could be detected through tissue than are
indicated in this assay.

35

EXAMPLE 5

*IN VIVO* DETECTION OF BIOLUMINESCENT *SALMONELLA*

To assess the availability of oxygen to *Salmonella* during infection, wild-type SL1344lux was inoculated into the
5  peritoneal cavity (i.p.) of BALB/c mice.  Photons emitted from the bacteria internally, and transmitted through the abdominal wall were externally detected and localized in anaesthetized mice using an intensified CCD camera 24 h after inoculation (Fig. 3B).  Systematic *Salmonella* infections are
10  thought to involve colonization of the lymph nodes, spleen, liver,.  Ventral images of the mice infected by i.p. inoculation of wild-type SL 1344lux demonstrated transmitted photons over much of the abdominal surface, with foci of various intensities (Fig. 3B).  These results were consistent
15  with widespread colonization of the viscera, possibly including the liver and mesenteric lymph nodes, and indicate that the level of available oxygen in some tissues can be adequate foe external detection of bioluminescence from the labelled pathogen.
20

EXAMPLE 6

DETECTION OF ORALLY-ADMINISTERED *LUX SALMONELLA* IN BALB/C MICE

Balb/c mice were infected by oral feeding (Stocker, *et al.*) with a 50 $\mu$l suspension of $1 \times 10^7$ virulent SL1344*lux*, non-
25  invasive BJ66*lux* and low virulence LB5000*lux Salmonella*.  The mice, 4-6 weeks of age at the time of infection, were imaged daily with 5 minute integration times (photon emission was measured for 5 minutes).  Prior to imaging, the mice were anesthetized with 33 $\mu$g/kg body weight nembutal.
30  Representative images are shown in Figures 5A-F.  At 24 hours post inoculation (p.i.), the bioluminescent signal localized to a single focus in all infected animals (Figs. 5A, 5C and 5E).  Bioluminescence disappeared in all animals infected with the low virulence LB5000*lux* by 7 days p.i.
35  (Fig. 5B).  In BALB/c mice infected with the wild-type SL1344lux, bioluminescence was detected throughout the study period, with multiple foci of transmitted photons at 8 d. In

these animals, the infection frequently spread over much of
the abdominal cavity (Fig. 5F).  In one-third of these
animals, transmitted photons were apparent over much of the
abdominal area at 8 d, resembling the distribution of photons
5  following an i.p. inoculation (see Figs. 3B and 5F).  The
spread of infection by BJ66lux was more variable, but the
infection typically persisted and remained localized at the
initial site (Fig. 5D).

    .After infection of resistant BALB/c x 129 mice with
10 wild-type SL 1344lux, the bioluminescent signal remained
   localized and persistent in a group of 10 mice throughout the
   study period.  This result was in contrast to the
   disseminated bioluminescence observed in SL1344lux-infected
   susceptible mice (lty$^{r/s}$) (see, Example 8 and Figs. 7A and
15 7B), but resembled the persistent infection of susceptible
   BALB/c mice with the less invasive BJ66lux.  As a control,
   *Salmonella* were cultured from persistently infected resistant
   BALB/c x 129 mice, and 80-90% of the colonies recovered after
   8 d were Amp$^{r}$.  Of these, more than 90% were bioluminescent,
20 suggesting that observed differences were not due to
   significant loss of *lux* plasmid, but rather were due to real
   differences in pathogenicity of the bacterial strains.

## EXAMPLE 7
25      DETECTION OF INFECTION FOLLOWING I.P. INOCULATION WITH A
             VIRULENT AND A LOW VIRULENCE STRAIN OF *SALMONELLA*

   Balb/c mice were infected with either virulent
   (SL1344*lux*) or low virulence (LB5000*lux*) *Salmonella* by
   intraperitoneal (i.p.) inoculations of 1 x 10$^7$ bacterial cells
30 in a 100 μl suspension, without simultaneous injection of
   air.

    At 32 hours post injection (p.i.), the mice were
   anesthetized and imaged as described above.  The results are
   shown in Figure 6.  Widespread infection is evident in the
35 two mice in the left part of the figure, infected with the
   virulent SL1344*lux* strain.  In contrast, little, if any,

luminescence is detected in the mice on the right, injected
with the low virulence LB5000*lux* strain.

### EXAMPLE 8

5      DETECTION OF SYSTEMIC INFECTION IN RESISTANT MICE FOLLOWING
              ORAL INOCULATION WITH *SALMONELLA*

Resistant 129xBalb/c (*Ity* $^{r/s}$) viable mice were infected
by intragastric inoculation of 1 x 10$^7$ SL1344*lux* *Salmonella*.
The bacteria were introduced through an intra-gastric feeding
10 tube while under anesthesia.  The animals were imaged daily
for 8 days post injection (d.p.i.).

Results are shown in Figures 7A and 7B.  Mice, in
triplicate, were infected and imaged daily for 8 days.
Exemplary images for day 1 (Fig. 7A) and day 8 (Fig. 7B) are
15 shown.  These data indicate that mice resistant to systemic
*Salmonella* infection have a localized chronic infection in
the cecum, but that the infection does not spread into the
abdominal cavity.

20                        EXAMPLE 9

        POST-LAPAROTOMY IMAGING FOLLOWING ORAL INOCULATION WITH *SALMONELLA*

Laparotomy was performed following oral inoculation of
*Salmonella* to precisely localize the luminescent signal
within the abdominal cavity, and to compare this localization
25 with that obtained using non-invasive imaging.  The animals
were inoculated as described in Example 8.  After a selected
period of time, typically seven days, the mice were
anesthetized and externally-imaged, as described above.  An
exemplary image is shown in Figure 8A.  After external
30 imaging, the peritoneal cavity was opened and the animals
were imaged again, as illustrated in Figure 8B.  In some
instances the mice were imaged a third time, following
injection of air into the lumen of the intestine both
anterior and posterior to the cecum (C) (Fig. 8C).  The mice
35 were euthanized immediately after the final imaging.

In each case where a focal pattern of bioluminescence
was observed in susceptible mice, early in infection after

oral inoculation, photons originated almost exclusively from
the cecum, while variations in the precise localization and
intensity of focal bioluminescence were due to variable
positioning of the cecum.  The focal pattern of

5 bioluminescence observed in infection-resistant BALB/c x 129
mice similarly localized to the cecum.  In contrast, such
localization was not observed in animals infected i.p. with
SL1344lux (Fig. 3B).  At late stages in infection-susceptible
mice inoculated orally with the wild-type SL1344lux,

10 bioluminescence was multifocal, however, additional foci of
luminescence did not become apparent after laparotomy.  In
mice infected with the less-virulent LB5000lux,
bioluminescence was not detectable at 7 d in any tissue or
organ, even focally, after removal of the skin and peritoneal

15 wall.

     Bioluminescence was not detected optically in the spleen
or bloodstream of any infected animal; bioluminescence from
the liver was seen only at later stages of disease; and
bioluminescence from the G.I. tract was restricted to the

20 cecum early in the disease course.  This pattern could be due
to differences in the numbers of *Salmonella* in the different
tissues, or lack of available oxygen.  The Amp$^r$ cfu present in
homogenized organs of orally infected mice were quantified to
evaluate the distribution of labelled *Salmonella* SL1344lux.

25 Greater than 90% of the amp$^r$ bacterial colonies obtained from
all analyzed tissues of SL1344lux-infected BALB/c mice at 7 d
indicated total cfu from the liver, spleen, and lungs were in
the range of 1.9 x 10$^3$ to > 1.0 x 10$^5$ without detectable
photon emission, *in vivo* (Table 2).  In contrast,

30 bioluminescence was detectable from the cecum and this tissue
contained > 1.0 x 10$^8$ total cfu.  No cfu were detectable in
any tissue of the LB5000lux infected mice.  These results
suggest that 1 x 10$^6$ organisms in tissue is near the limit of
detection at this emission wavelength using the current

35 experimental system.

     Oxygen is an essential substrate for the luciferase
reaction, thus only *Salmonella* present in oxygenated

microenvironments should be bioluminescent.  The absence of
bioluminescence from *Salmonella* in the anaerobic environment
of the lumen of the G.I. tract is therefore predictable, and
exposure of the intestinal lumen to air should reveal the
5 presence of bacteria previously not detectable due to a lack
of oxygen.  In support of this view, one animal with
detectable bioluminescence in the cecum alone excreted a
faecal pellet that rapidly became bioluminescent upon
exposure to air.  This indication of non-luminescent,
10 luciferase-expressing bacteria in the lumen of the intestine
and the clear delineation of the aerobic and anaerobic zones
in this tissue, suggested that injection of air into the
lumen of the intestine would reveal the presence of
additional bacteria.  Injection of air into the lumen of the
15 ileum and colon of another animal, with a similar pattern of
bioluminescence, resulted in detectable photons near the
injection sites (Fig. 8).  Last, when a third mouse with
cecal bioluminescence was killed, bioluminescence quickly
ceased.  Air was injected at other tissue sites because of
20 the lack of clear zones of aerobic and anaerobic
environments.

25

30

35

TABLE 2

Colony-forming units in homogenized tissue from mice infected
with bioluminescent *Salmonella*

| Strain | Tissue | Animal Number | Tissue Weight (mg) | Total cfu |
|---|---|---|---|---|
| SL1344lux | Liver | 1 | 441 | $1.9 \times 10^3$ |
| | | 2 | 778 | $2.5 \times 10^4$ |
| | Spleen | 1 | 218 | $1.2 \times 10^4$ |
| | | 2 | 248 | $4.9 \times 10^5$ |
| | Mesenteric lymph node | 1 | 76 | $>1.0 \times 10^6$ |
| | | 2 | 46 | $>1.0 \times 10^6$ |
| | Lung | 1 | 17 | $1.5 \times 10^3$ |
| | | 2 | 69 | $2.7 \times 10^3$ |
| | Cecum | 1 | 351 | $>1.0 \times 10^{8*}$ |
| | | 2 | 422 | $>1.0 \times 10^{8*}$ |

*. Photons emitted from bacteria at these tissue sites were
externally detected.

EXAMPLE 10

POST-LAPAROTOMY IMAGING FOLLOWING I.P. INOCULATION WITH *SALMONELLA*

Balb/c mice were infected by intraperitoneal inoculation
of $1 \times 10^7$ *Salmonella* (SL1344lux) as described in Example 7.
Exemplary images of one such animal are shown in Figures 9A,
9B and 9C.

At 24 hours post-injection (p.i.), the animal was
anesthetized and imaged for five minutes (Figure 9A). The
peritoneal cavity was opened and the mouse was imaged again
for five minutes (Figure 9B). The cecum was pulled to the
left side, and the animal was again imaged for five minutes
(Figure 9A).

The results demonstrate that the localization of
infection sites obtained with non-invasive imaging correlates
well with the sites as revealed upon opening the peritoneal
cavity.

EXAMPLE 11

<u>EFFECTS OF CIPROFLOXACIN TREATMENT ON BIOLUMINESCENCE</u>

<u>FROM SL1344LUX *SALMONELLA*</u>

To demonstrate the utility of *in vivo* imaging, an

5 infected animal was treated with the antibiotic

ciprofloxacin, which known to be effective against systemic

*Salmonella* infections.  Magalianes, *et al.*, 1993,

*Antimicrobial Agents Chemo.* <u>37</u>:2293.

Experimental and control groups of Balb/c mice were

10 orally inoculated with SL1344*lux*.  At 8 days p.i.,  mice in

the experimental group were injected i.p. with 100 mg of

ciprofloxacin hydrochloride (3mg/kg body weight; Sigma

Chemical Co., St. Louis, MO).  Following treatment of the

experimental group, animals from both groups were imaged (as

15 above) at several intervals over a period of 5.5 h post

treatment.

Representative images are shown in Figures 10B-E.

Figures 10B and 10D show composite images of representative

animals from the control and treated groups, respectively,

20 immediately before initiation of treatment of the

experimental group.  Figures 10C and 10E show composite

images of the same animals 5.5 hours after initiation of

treatment.  Bioluminescence over the abdomen of the

ciprofloxacin-treated animal was reduced to undetectable

25 levels during this period of time, while bioluminescence in

the control typically increased 7.5-fold.  The total number

of photons detected over the abdominal area were determined,

normalized to the value at t=0, and plotted in Figure 10A

with respect to time post-treatment.

30    The data demonstrate that methods and compositions of

the present invention can be used to evaluate the effects of

drugs on the spread of infection *in vivo*.

While the invention has been described with reference to

35 specific methods and embodiments, it will be appreciated that

various modifications and changes may be made without

departing from the invention.

WHAT IS CLAIMED IS:

1.   A noninvasive method for detecting the localization
of a biocompatible entity in a mammalian subject, comprising
5        (a) administering to the subject a conjugate of the
entity and a light-generating moiety,
        (b) after a period of time in which the conjugate can
achieve localization in the subject, immobilizing the subject
within the detection field of a photodetector device,
10        (c) maintaining the subject in an immobilized condition,
        (d) during said maintaining, measuring photon emission
from the light-generating moiety, localized in the subject,
with the photodetector device until an image of photon
emission can be constructed, and
15        (e) constructing such an image.


2.   The method of claim 1, which further includes
repeating steps (b) through (e) at selected intervals,
        wherein said repeating is effective to track the
20 localization of the entity in the subject over time.


3.   The method of claim 1, where said measuring is
carried out with an intensified charge-coupled photodetector
device.
25

4.   The method of claim 1, for detecting the
localization of tumor cells in a mammalian subject, where
said administering includes administering a conjugate that
contains a tumor cell targeting moiety.
30

5.   The method of claim 1, for detecting the
localization of inflammation in a mammalian subject, where
said administering includes administering a conjugate that
contains an inflammation-targeting moiety.
35

6.   The method of claim 1, for detecting the
localization of infection by a pathogen in a mammalian

- 64 -

subject, where said administering includes administering a
conjugate that contains an infection-targeting moiety.

7.   The method of claim 1, for detecting the
5  localization of thrombotic plaques in a mammalian subject,
where said administering includes administering a conjugate
that contains a thrombotic plaque-targeting moiety.

8.   The method of claim 1, where said administering
10 includes administering a conjugate that is a particle
containing a light-generating moiety.

9.   The method of claim 1, for detecting the
localization of infection by a pathogen in an animal model,
15 where the bio-compatible entity is the pathogen.

10.   The method of claim 9, where the pathogen is
_Salmonella_.

20     11.   The method of claim 1, where the light-generating
moiety is a light-generating protein.

12.   The method of claim 11, where the protein is
selected from the group consisting of luciferase, yellow
25 fluorescent protein and ferredoxin IV.

13.   The method of claim 11, where said administering
includes administering a conjugate that is a fusion protein
of an antibody fragment and a light-generating protein.
30
14.   The method of claim 11, where the biocompatible
entity is a transformed cell, and the light-generating moiety
is a product of a heterologous gene expressed by the cell.

35     15.   The method of claim 14, where expression of the
heterologous gene is under the control of an activatable
promoter.

16.  A noninvasive method for detecting the level of a
biocompatible entity in a mammalian subject over time,
comprising
        (a) administering to the subject a conjugate of the
5 entity and a light-generating moiety,
        (b) placing the subject within the detection field of a
photodetector device,
        (c) maintaining the subject in the detection field of
the device,
10      (d) during said maintaining, measuring photon emission
from the light-generating moiety, in the subject, with the
photodetector device, and
        (e) repeating steps (b) through (d) at selected
intervals, wherein said repeating is effective to detect
15 changes in the level of the entity in the subject over time.


        17.  A noninvasive method for detecting the integration
of a transgene in a mammalian subject, comprising
        (a) administering to the subject, a vector construct
20 effective to integrate into mammalian cells and containing a
transgene, a gene encoding a light-generating protein, and an
activatable promoter, said gene encoding a light-generating
protein being under the control of said promoter,
        (b) after a period of time in which the construct can
25 achieve integration, activating said promoter,
        (c) placing the subject within the detection field of a
photodetector device,
        (d) maintaining the subject within the detection field
of the photodetector device,
30      (e) during said maintaining, measuring the level of
photon emission from expressed light-generating protein, in
the subject, with the photodetector device, and
        (f) confirming integration of the transgene if the level
of photon emission is significantly higher than background.
35

        18.  A noninvasive method for detecting the localization
of a promoter-induction event in an animal, comprising

(a) triggering the event in a transgenic animal having an inducible promoter responsive to such event and a heterologous gene encoding a light-generating protein under control thereof,

5      (b) placing the animal in the detection field of a photodetector device,

(c) maintaining the animal in an immobilized condition,

(d) during said maintaining, measuring photon emission from expressed light-generating protein, localized in the 10 animal, with the photodetector device until an image of photon emission can be constructed, and

(e) constructing such an image.

19.   *Salmonella* transformed with a gene expressing a 15 light-generating protein.

20

25

30

35

1/18
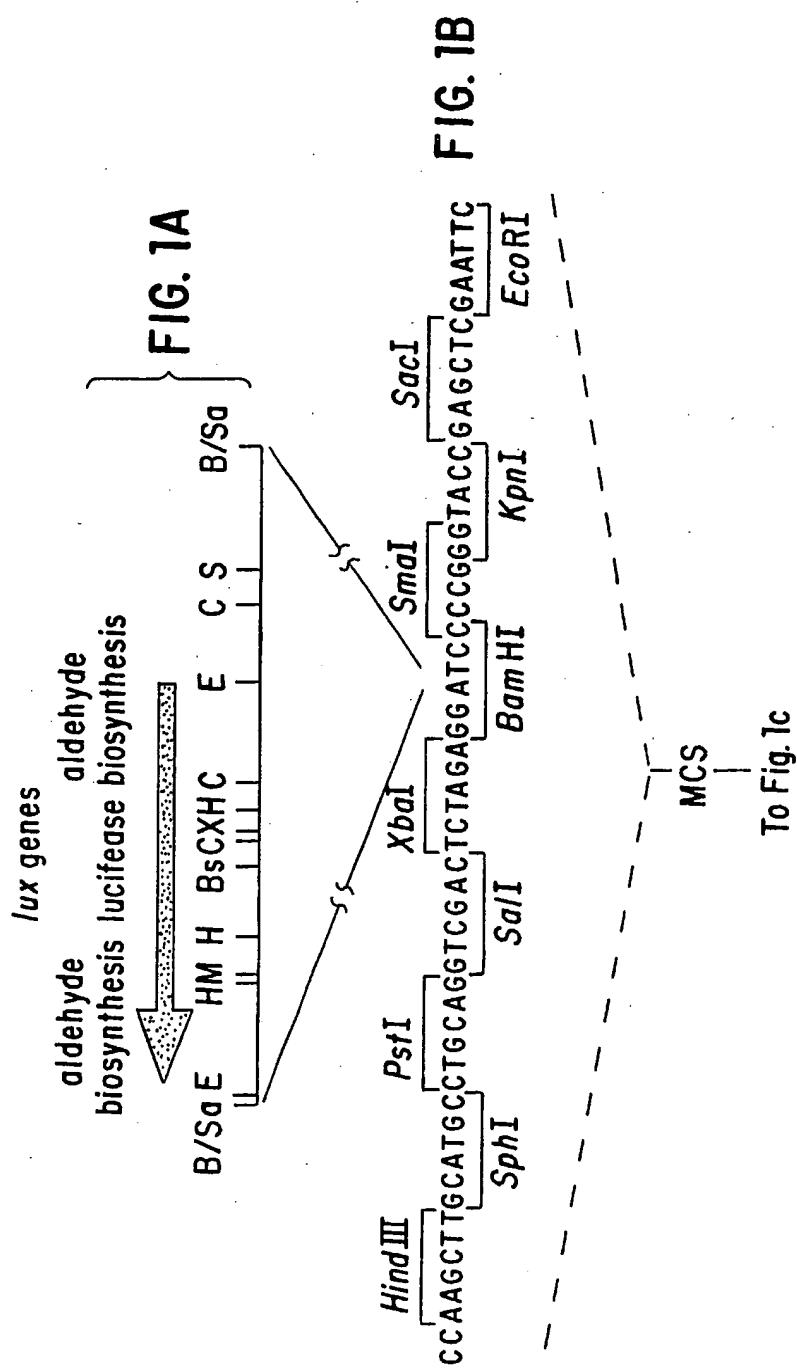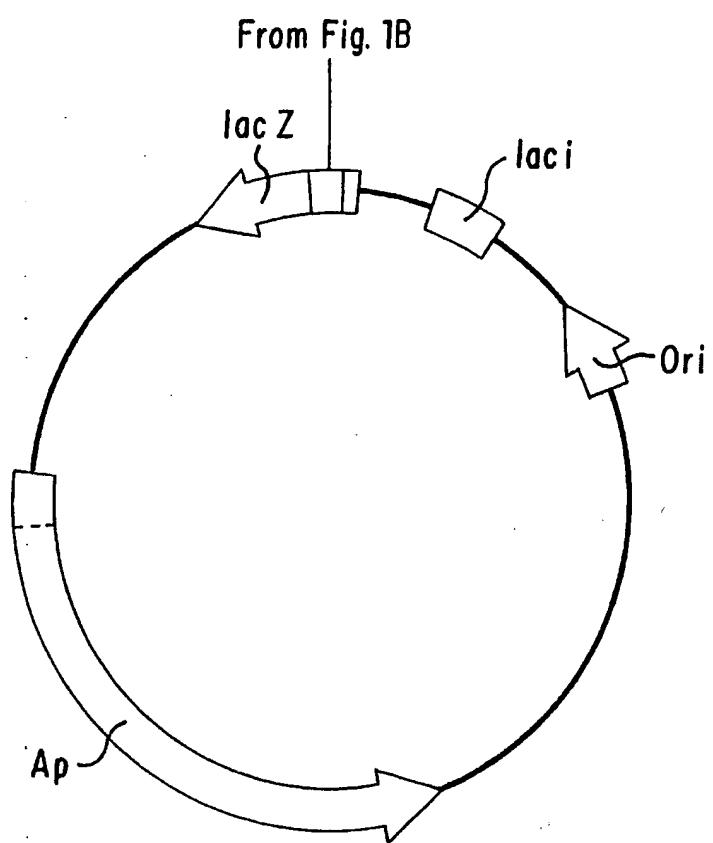


FIG. 1A

FIG. 1B

From Fig. 1B

lac Z

lac i

Ori

Ap

FIG. 1C

FIG.2A

FIG. 2D

FIG. 2E

FIG. 2B

FIG. 2C

FIG.3A

1d
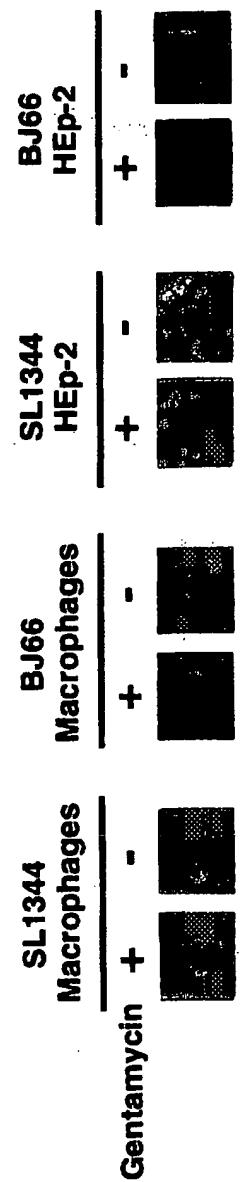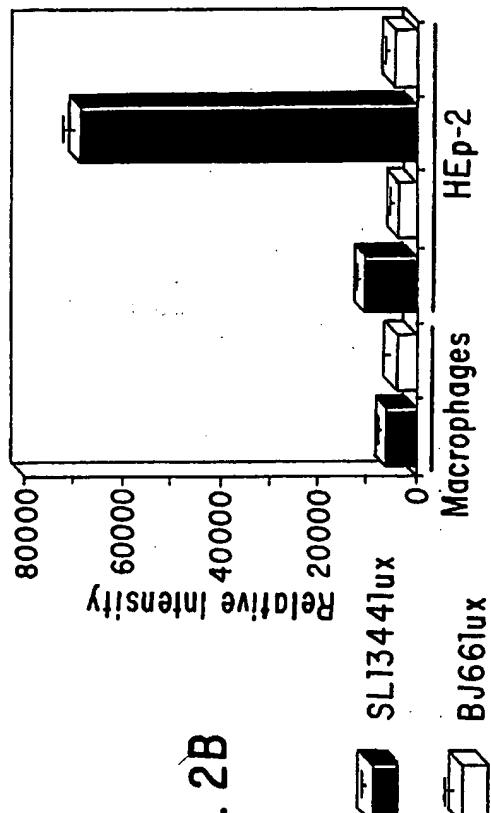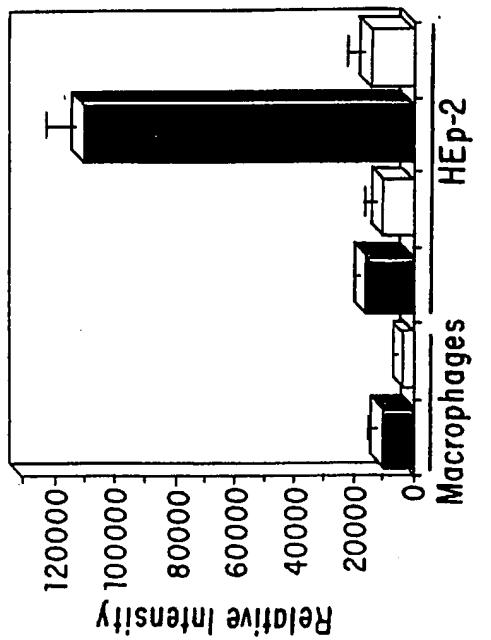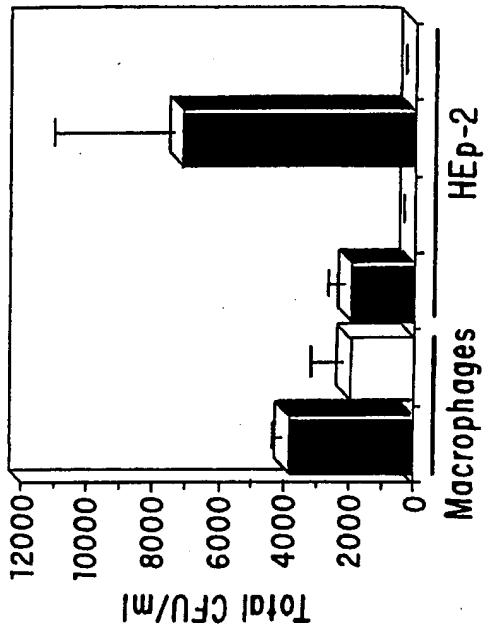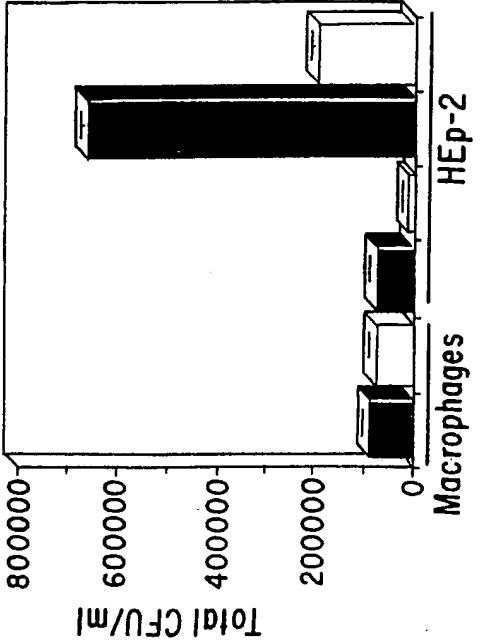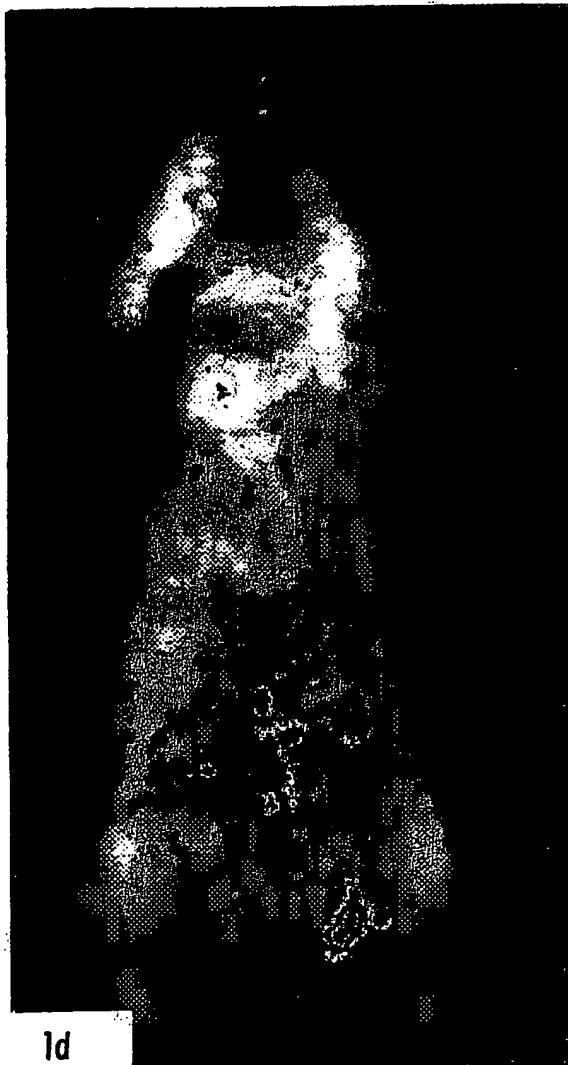
SL 1344 lux
(intraperitoneal)
FIG.3B
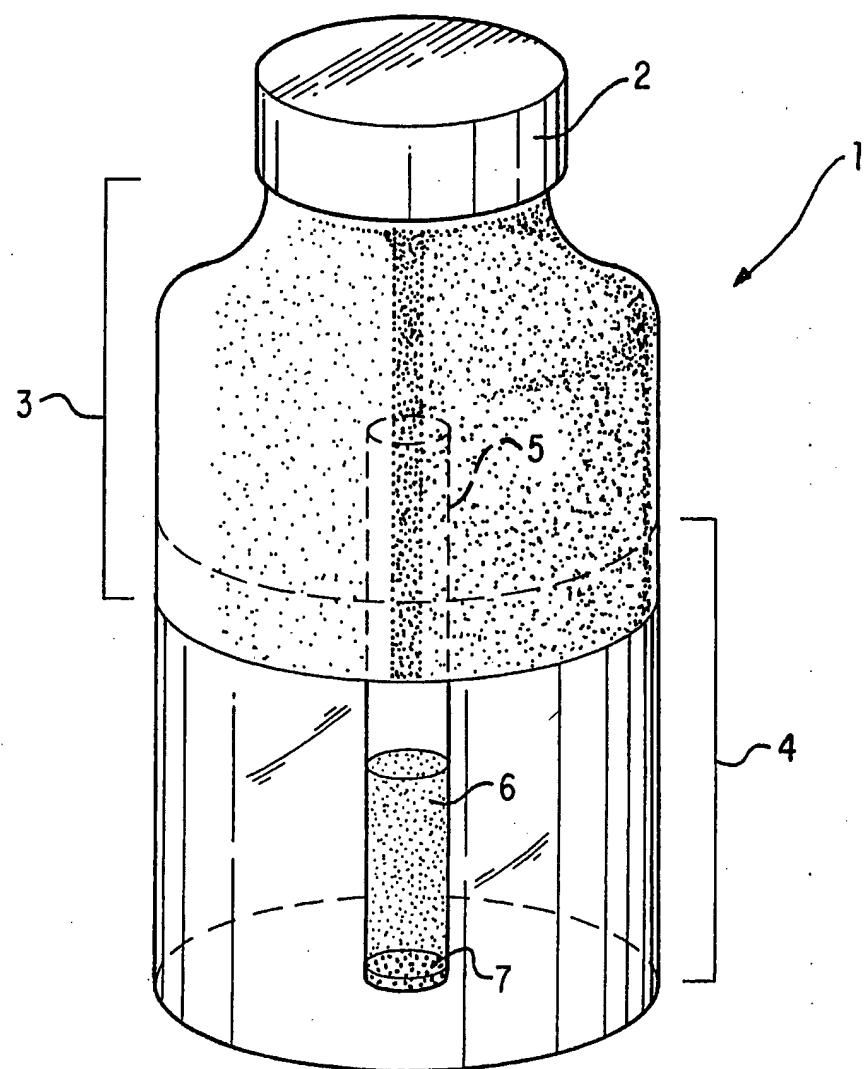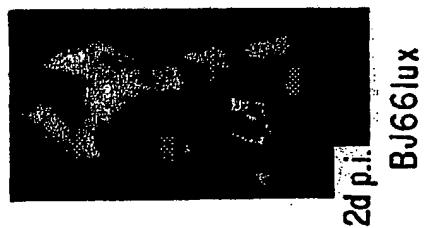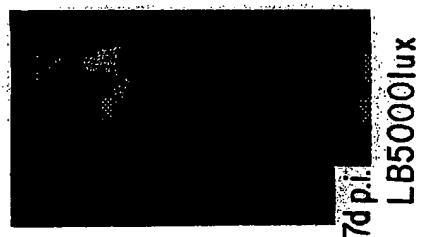
FIG. 4

8/18



2d p.i.
BJ66lux

FIG.5C



7d p.i.
LB5000lux

FIG.5B



1d p.i.
LB5000lux

FIG.5A

8d.p.i.
SL1344lux

FIG.5F

2d.p.i.
SL1344lux
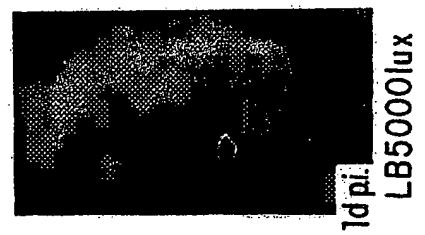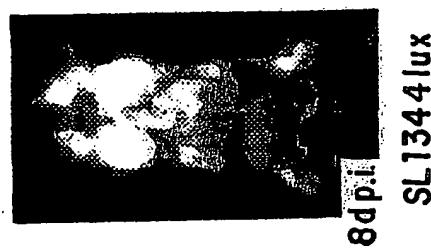
FIG.5E
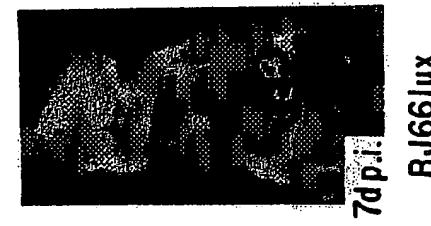
7d.p.i.
BJ66lux

FIG.5D
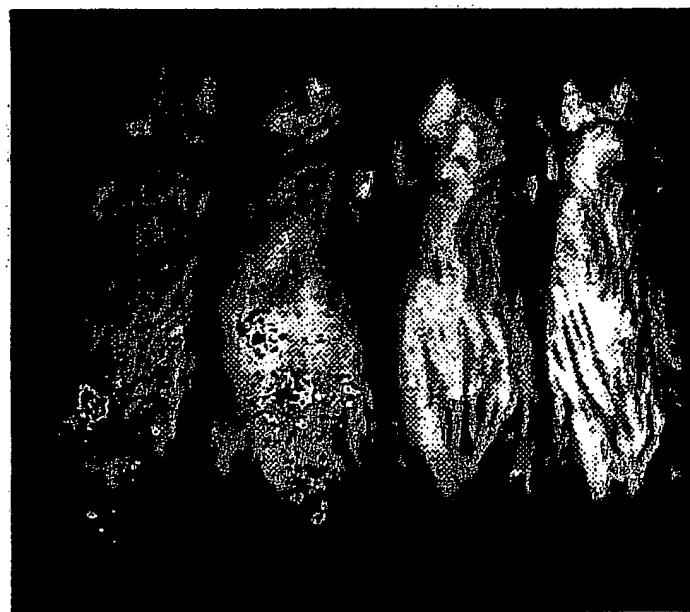
FIG.6

FIG.7A



FIG.7B
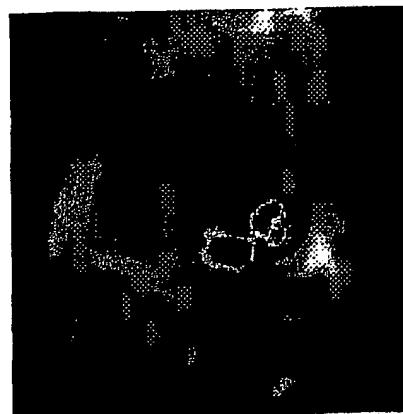
**BJ66lux Infection (7d p.i.)**

# FIG.8A
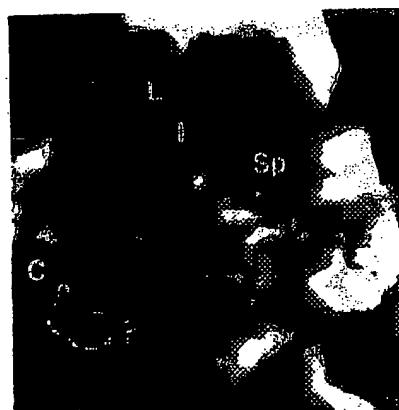


**BJ66lux Infection (7d p.i.)**

# FIG.8B

BJ66lux Infection (7 d p.i.)

FIG.8C
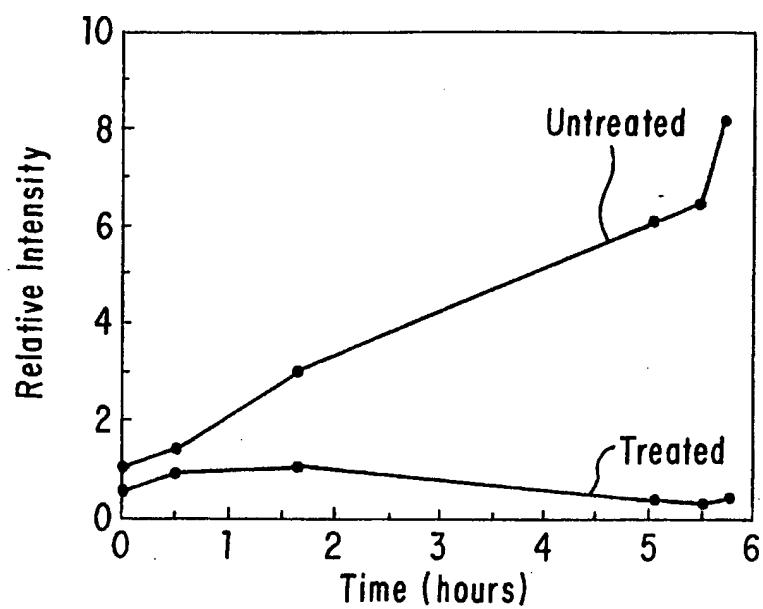
FIG.9A



FIG.9B

FIG.9C

FIG. 10A

17/18
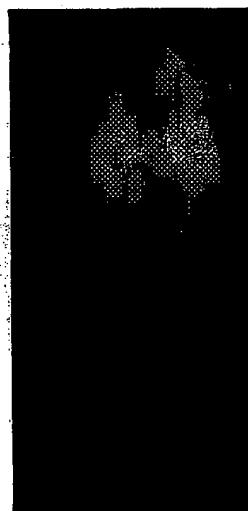


FIG.10B



FIG.10C

FIG.10D



FIG.10E

# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER
IPC 6    A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6    A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | MOL. MICROBIOL. (1995), 18(4), 593-603 CODEN: MOMIEE;ISSN: 0950-382X, 1995, XP002010241 CONTAG, CHRISTOPHER H. ET AL: "Photonic detection of bacterial pathogens in living hosts" | 1-19 |
| Y | see the whole document --- -/-- | 1-3,5 |

[X] Further documents are listed in the continuation of box C.    [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 6 August 1996 | 2 0. 08. 96 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016 | Berte, M |

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | CHEMICAL ABSTRACTS, vol. 123, no. 15, 9 October 1995 Columbus, Ohio, US; abstract no. 189418, ARAKI, KAZUO: "Photo-detection of transferred gene expression in fish" XP002010244 see abstract & SEIBUTSU BUTSURI (1995), 35(3), 119-20 CODEN: SEBUAL;ISSN: 0582-4052, 1995, | 1,16-18 |
| Y | METHODS IN CELL BIOLOGY, vol. 33, 1 January 1990, pages 469-490, XP000376615 HORAN P K ET AL: "FLUORESCENT CELL LABELING FOR IN VIVO AND IN VITRO CELL TRACKING" see page 472, paragraph 4 | 1-3,5 |
| X | DATABASE BIOSIS BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US AN=95:237442, XP002010246 see abstract & PEDIATRIC RESEARCH, vol. 37, no. 4PART2, 1994, page 172A CONTAG C.H. ET AL.: "OPTICAL TRACKING OF INFECTION IN VIVO USING LIGHT-GENERATING PROBES." | 1-4,6,9, 10,16 |
| A | ANGEWANDTE CHEMIE INTERNATIONAL EDITION., vol. 33, 1994, WEINHEIM DE, pages 1044-1072, XP002010242 A. MAYER ET AL.: "LUMINESCENT LABELS-MORE THAN JUST AN ALTERNATIVE TO RADIOISOTOPES?" see page 1054, column 2 | 1-12 |
| A | WO,A,86 01806 (TECHNOLOGY LICENCE CO LTD) 27 March 1986 see page 8, line 18 - line 32; claims 1,20,21,28,30,38,39 | 1-3,6,9, 11,12 |
| A | WO,A,86 01805 (TECHNOLOGY LICENCE CO LTD) 27 March 1986 see claims 1,24,25,32,33 | 1,6,9-12 |

-/--

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|---|---|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | SCIENCE,<br>vol. 259, 5 March 1993, LANCASTER, PA US,<br>pages 1463-1466, XP002010243<br>D.A. BENARON ET AL.: "OPTICAL<br>TIME-OF-FLIGHT AND ABSORBANCE IMAGING OF<br>BIOLOGICAL MEDIA."<br>see figure 3<br>--- | 1-3 |
| A | EP,A,0 263 657 (CIBA CORNING DIAGNOSTICS<br>CORP) 13 April 1988<br>see claims<br>--- | 1 |
| X | CHEMICAL ABSTRACTS, vol. 121, no. 25,<br>19 December 1994<br>Columbus, Ohio, US;<br>abstract no. 297047,<br>MACKEY, B.M. ET AL: "Thermostability of<br>bacterial luciferase expressed in<br>different microbes"<br>XP002010245<br>see abstract<br>& J. APPL. BACTERIOL. (1994), 77(2),<br>149-54 CODEN: JABAA4;ISSN: 0021-8847,<br>1994,<br>--- | 19 |
| X | WO,A,91 01305 (UNIV WALES MEDICINE) 7<br>February 1991<br>see page 7, line 2 - page 8, line 2;<br>claims 1,15<br>----- | 1,3,6,9,<br>11-15 |

1

**Box I    Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

**Please see Further Information sheet enclosed.**

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II    Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**      [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

**FURTHER INFORMATION CONTINUED FROM**    PCT/ISA/210

Remark : Although claims 1 - 18 are directed to a method of treatment of
(diagnostic method practised on) the human/animal body the search
has been carried out and based on the alleged effects of the
compound/composition.

# INTERNATIONAL SEARCH REPORT

...formation on patent family members

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-8601806 | 27-03-86 | EP-A- | 0192726 | 03-09-86 |
| | | JP-T- | 62500173 | 22-01-87 |
| WO-A-8601805 | 27-03-86 | EP-A- | 0193576 | 10-09-86 |
| | | JP-T- | 62500172 | 22-01-87 |
| EP-A-0263657 | 13-04-88 | US-A- | 4745181 | 17-05-88 |
| | | AU-B- | 595644 | 05-04-90 |
| | | AU-B- | 7910087 | 14-04-88 |
| | | DE-A- | 3779040 | 17-06-92 |
| | | JP-B- | 7002716 | 18-01-95 |
| | | JP-A- | 63101368 | 06-05-88 |
| | | US-A- | 4918192 | 17-04-90 |
| | | US-A- | 5110932 | 05-05-92 |
| WO-A-9101305 | 07-02-91 | AU-B- | 6054590 | 22-02-91 |
| | | EP-A- | 0484369 | 13-05-92 |
| | | JP-T- | 5501862 | 08-04-93 |